Binding of hirudin to meizothrombin

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Prothrombin (coagulation factor II) is the inactive precursor molecule of thrombin (coagulation factor IIa). Proteolytic cleavage of the peptide bond Arg320–Ile321 converts prothrombin into the two-chain thrombin precursor meizothrombin. Meizothrombin hydrolyses peptideyl substrates, but cleavage of fibrinogen is poor. Unfortunately, meizothrombin exhibits a significant autocalycytic activity and thus is not structurally stable in solution. Hirudin, the 65-residue peptide anticoagulant from the salivary gland of the European leech Hirudo medicinalis, is a highly specific and effective thrombin inhibitor. To study the interactions of meizothrombin and hirudin, recombinant prothrombin with active site Asp419 replaced by Asn (D419N-prothrombin) was produced in CHO cells and transformed into D419N-meizothrombin in vitro. D419N-meizothrombin exhibited no proteolytic and autocalycytic activity. D419N-meizothrombin was affinity purified at an immobilized C-terminal hirudin-derived peptide demonstrating the presence and activity of the anion binding exosite. D419N-meizothrombin exhibited binding activity to hirudin immobilized at the solid phase in an ELISA. Incubation of D419N-meizothrombin with hirudin resulted in a significant increase of intrinsic fluorescence. Fluorescence titration of D419N-meizothrombin with hirudin produced a sharp break in the titration curve at the molar equivalence point and a total fluorescence enhancement of 24%. However, the titration curve did not reflect a simple binding mechanism. Incubation of D419N-meizothrombin with fibrinopeptide A and C-terminal hirudin peptide 54–65 did not change fluorescence emission. Trp468 located in the γ-loop of thrombin was replaced by Phe in the double-mutant D419N/W468F-thrombin. Similar to D419N-thrombin and D419N-meizothrombin, formation of the D419N/W468F-thrombin/hirudin complex resulted in a significant increase in intrinsic fluorescence. Apparently, the binding of hirudin induces similar structural changes in both meizothrombin and thrombin. The structural change does not involve the flexible γ-loop. The results suggest that meizothrombin binds hirudin similar to thrombin.

Keywords: hirudin/prothrombin/thrombin/meizothrombin/recombinant protein/coagulation factor

Introduction

Human prothrombin (coagulation factor II) is the single polypeptide chain precursor of the blood clotting proteinase thrombin (coagulation factor IIa). Prothrombin contains 579 amino acid residues (for amino acid numbering the prothrombin amino acid numbers are used consequently) and is composed of several distinct domains: fragment 1 (residues 1–155), fragment 2 (residues 156–271), thrombin A-chain (residues 272–320) and thrombin B-chain (residues 321–579). Thrombin is the activation product of prothrombin that initiates the final stages of blood coagulation. It is a serine protease that cleaves the fibrinogen molecule into fibrin monomers and activates coagulation factor XIII, which then catalyses fibrin crosslinking reactions. The catalytic centre of thrombin is contained in the B-chain and comprises the amino acids His363, Asp419 and Ser525. Thrombin also controls other reactions in the blood clotting system by a feed-back mechanism and is a highly potent stimulant of platelet release (Lundlad et al., 1977). In vivo thrombin is liberated when prothrombin is activated by Factor Xa, Factor V and calcium ions (Jackson and Nemerson, 1980) by hydrolysis of the Arg271–Thr272 and Arg320–Ile321 peptide bonds yielding thrombin composed of thrombin A-chain and B-chain. However, when cleavage of Arg320–Ile321 bonds precedes hydrolysis of the Arg271–Thr272 bond, meizothrombin is formed composed of two peptide chains, Ala1–Arg320 and Ile321–Glu579, linked by a single disulphide bond. Meizothrombin hydrolyses low molecular peptide substrates at comparable rates to thrombin (Doyle and Haley, 1993), demonstrating that the catalytic centre is already formed and that substrate binding sites at and close to the catalytic centre are active. By contrast, cleavage of fibrinogen by meizothrombin is very poor (Doyle and Haley, 1993). It is a common belief that the inactivity of meizothrombin towards physiologic substrates such as fibrinogen is caused by the presence of the F1/F2-peptide which may alter the enzyme specificity and/or block fibrinogen from binding to the anion binding exosite. Hirudin, the 65-residue peptide anticoagulant from the salivary gland of the European leech, Hirudo medicinalis, is the most specific and most effective inhibitor of the blood protease thrombin (Markwardt, 1991a,b). The hirudin–thrombin complex has a very low equilibrium dissociation constant in the fM range. From this complex, hirudin may only be liberated by heat denaturation of thrombin (Markwardt and Walsmann, 1958). Hirudin has received attention recently as a potent therapeuic agent for the control of thrombosis. Based on pharmacological and clinical profiling of recombinant hirudin, clinical indications such as surgical anticoagulation, medical anticoagulation, adjunctive administration with thrombolytic agents, anticoagulation during percutaneous transluminal coronary angioplasty, postoperative thrombosis prophylaxis, prevention of rethrombosis, microvascular surgery and anticoagulant in hemodialysis and extracorporal circulation are explored (Bichler and Fritz, 1991; Fareed et al., 1991; Johnson, 1994; Lefkovits and Topol, 1994; Markwardt, 1994).

The interactions of thrombin and hirudin have been studied in great detail. The molecular basis of the hirudin–thrombin binding includes interactions of the anion binding exosite of...
the thrombin molecule and the C-terminus of hirudin, and interactions of the active-site cleft of the enzyme and the N-terminus of the inhibitor. However, hirudin does not occupy the S1 substrate binding subsite of thrombin and makes no major contact to the catalytic triad (Rydel et al., 1990, 1991). By contrast, interaction(s) of meizothrombin and hirudin have not been studied in detail. This results from instability of meizothrombin which exhibits significant autocatalytic activity. Even in the presence of inhibitors the stability of transient prothrombin activation products is low (Doyle and Mann, 1990).

Previous studies demonstrated that binding of hirudin to human plasma-derived α-thrombin results in an increase in intrinsic fluorescence emission (Jackman et al., 1992; Parry et al., 1993). However, even highly purified human α-thrombin preparations may contain contaminations of other structurally related coagulation factors and active blood proteases, which can interfere with the assay system. Prothrombin-derived molecules, such as α-thrombin, exhibit a notable autocatalytic potential and are not stable in solution and are converted into molecules such as γ-thrombin and smaller degradation products. Therefore, these molecules are not useful for equilibrium titration experiments.

Crystallographic analysis of the catalytic centre of human thrombin showed that Asp419 does not contribute to the binding of hirudin (Rydel et al., 1990, 1991). To generate autocatalytic and proteolytically inactive but structurally stable thrombin-derivatives with active inhibitor binding sites, we have exchanged Asp419 of the catalytic centre with Asn. Recombinant D419N-prothrombin was expressed in CHO cells and converted in vitro into D419N-thrombin and D419N-meizothrombin (Fischer et al., 1996a). For comparison, r-prothrombin was produced and converted to r-thrombin. In contrast to r-thrombin, D419N-thrombin and D419N-meizothrombin exhibited no clotting and no autocatalytic activities (Fischer et al., 1996a,b). Both D419N-thrombin and D419N-meizothrombin have been used successfully as hirudin antagonists for the neutralization of hirudin in vivo and in vitro (Fischer et al., 1996a).

In the present study the interaction of hirudin with D419N-meizothrombin, a structurally stable modification of human meizothrombin, was studied by fluorescence spectroscopy. The results were compared with fluorescence studies of D419N-thrombin and r-thrombin.

Materials and methods

Materials

Recombinant hirudin (desulfato hirudin variant HV1) was produced by yeast strains of Hansenula polymorpha (Fischer et al., 1996a; Fischer et al., 1996b,c,d). r-Prothrombin, D419N-prothrombin and D419N/W468N-prothrombin were purified from a cell culture supernatant of transformed CHO cells by combination of anion exchange chromatography and calcium ion-dependent filtration as described previously (Fischer et al., 1995a). Conversion of r-prothrombin, D419N-prothrombin and D419N/W468N-prothrombin into r-thrombin, D419N-thrombin and D419N/W468F-thrombin, respectively, and conversion of D419N-prothrombin into D419N-meizothrombin was performed by incubation of the proteins in 20 mmol/l Tris–HCl buffer, pH 7.4, 1.50 mmol/l NaCl, 10 mmol/l CaCl2, with Oxyuranus scutellatus venom and Echis carinatus venom, at a prothrombin/venom ratio of 50:1 at 4°C for 20 h as described previously (Fischer et al., 1996b). r-Thrombin, D419N-thrombin, D419N/W468F-thrombin and D419N-meizothrombin were purified from the activation mixtures by affinity chromatography as described previously (Fischer et al., 1996d).

Briefly, the synthetic peptide NH2-Lys-Pro-Gly-Pro-Gly-Ser-Ala-Asp-Gly-Asp-Phe-Glu-Ile-Pro-Glu-Tyr-Leu-COOH was covalently linked with NHS-activated Sepharose 4 Fast Flow yielding a new affinity matrix specific for the thrombin anion binding exosite. The activation mixtures were filtered through the affinity matrix and washed with 20 mmol/l Tris–HCl buffer, 150 mmol/l NaCl, pH 7.4. Bound protein was eluted with 1.2 mol/l KSCN in Tris–HCl buffer and immediately desalted.

Analytical methods

Protein concentration of thrombin and meizothrombin was determined photometrically at 280 nm using extinction coefficients (1 mg/ml, d = 1 cm) of 1.83 and 1.32, respectively (Haeberli, 1992). Hirudin concentration was determined photometrically at 275 nm using the molar extinction coefficient of 3099 M–1 cm–1 (Rowand and Berliner, 1992). Clotting activity of prothrombin was determined by a one-step prothrombin time test as described in detail previously (Fischer et al., 1995a) using a prothrombin concentration standard to prepare the standard curve. Thrombin activity was determined photometrically by the hydrolysis of the synthetic thrombin-specific chromogenic substrate AcOHD-CHG-Ala-Arg-pNA at 37°C in 50 mM Tris–HCl buffer, pH 8.0, containing 150 mM NaCl, 0.1% PEG 6000 and a final substrate concentration of 200 nM. The release of p-nitroaniline that resulted from the hydrolysis of the peptidyl p-nitroanilide substrate was measured by monitoring the increase in absorbance at 410 nm. A thrombin concentration standard was used to prepare the calibration curve. Active site concentration of thrombin derivatives and meizothrombin was titrated using 4-methylumbelliferyl-p-guanidinobenzoate (Doyle and Haly, 1993). Electrophoretic analysis of protein was performed at reducing conditions by SDS–PAGE in 10% acrylamide slab gels using the buffer system described by Laemmli (1970).

Binding of r-thrombin, D419N-thrombin and D419N-meizothrombin to immobilized hirudin

Binding of r-thrombin, D419N-thrombin and D419N-meizothrombin to immobilized hirudin in an ELISA has been described in detail previously (Fischer et al., 1995b). Briefly, 100 µl r-hirudin (2 µg/ml in 50 mM carbonate buffer pH 9.6) was coated onto microtiter plates at 4°C for 16 h. Microtiter plate wells were incubated with 100 µl of sample material containing r-thrombin, D419N-thrombin or D419N-meizothrombin for 60 min. Hirudin-bound protein was quantitatively detected by incubation with 100 µl solution of affinity-purified sheep anti-human thrombin IgG–peroxidase conjugate.
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Fig. 1. Affinity purification of (A) r-thrombin, (B) D419N-thrombin, (C) D419N-meizothrombin and (D) D419N/W468F-thrombin. The reduced proteins were run on SDS–polyacrylamide gels (10%) and stained with Coomassie blue. Lane A: (a) molecular weight marker; (b) r-prothrombin; (c) r-thrombin. Lane B: (a) molecular weight marker; (b) D419N-prothrombin; (c) D419N-thrombin. Lane C: (a) molecular weight marker; (b) D419N-prothrombin; (c) D419N-meizothrombin. Lane D: (a) molecular weight marker; (b) D419N/W468F-prothrombin; (c) D419N/W468F-thrombin. Molecular weight marker (from top to bottom): 116 300; 97 400; 66 300; 55 400; 36 500; 31 000.

followed by peroxidase colour development. Colour intensity at 450 nm was measured by an automated plate reader (absorbance at 450 nm). Colour intensity data were corrected for background readings.

Fluorescence studies

Fluorescence studies were performed using a Perkin-Elmer LS50 fluorescence spectrophotometer and 1.0 × 1.0 cm quartz cuvettes equilibrated at 25°C. Fluorescence studies were performed in 50 mM Tris–HCl buffer, pH 7.4, containing 150 mM NaCl and 0.1% PEG 6000. The fluorescence equilibrium titration studies of r-thrombin, D419N-thrombin, D419N-meizothrombin and D419N/W468F-thrombin with hirudin, C-terminal hirudin peptides and fibrinogen peptide A were performed by sequential addition of 1 µl of hirudin, C-terminal hirudin peptide and fibrinogen peptide A, respectively, to 2000 µl of protein solution. Excitation and emission wavelengths of 280 nm (band width 2.5 nm) and 341 nm (band width 5 nm) were used, respectively. Dilution effects were neglected as the volume increase after 10 aliquots was only 0.5%. For fluorescence emission spectra the excitation wavelength was 280 nm (band width 2.5 nm) and fluorescence emission was measured between 300 and 400 nm (band width 2.5 nm). Fluorescence date were corrected for background readings.

Results

r-Prothrombin and D419N-prothrombin were purified from cell culture of transformed CHO cells. r-Prothrombin and D419N-prothrombin were converted into r-thrombin and D419N-thrombin, respectively, by limited proteolysis. Additionally, D419N-prothrombin was converted into D419N-meizothrombin. r-Thrombin, D419N-thrombin and D419N-meizothrombin were affinity purified (Figure 1) using an immobilized peptide with the amino acid sequence NH2-Lys-Pro-Gly-Pro-Gly-Ser-His-Ala-Asp-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-COOH. This peptide corresponds to the C-terminal part of hirudin (amino acid 46–65) with the modification that an additional Lys has been added at the N-terminus and that Lys47, Gln49 and Asn52 of hirudin have been replaced by Gly, Gly and Ala, respectively, to ensure immobilization of the peptide to Sepharose exclusively via its N-terminal amino groups (Fischer et al., 1996a,d).

While D419N-thrombin and D419N-meizothrombin exhibited no notable activity towards the thrombin-specific chromogenic peptide substrates 2AcOH.H.D-CHG-Ala-Arg-pNA, r-thrombin exhibited a specific activity of 98.4 nmol/min/µg protein. Active site titration of r-thrombin resulted in 16.34 nmol/mg protein. No active site was detected for D419N-thrombin and D419N-meizothrombin.

To test whether D419N-thrombin, D419N-meizothrombin and r-thrombin exhibit hirudin binding activity, hirudin was immobilized at the solid phase of microtiter plates and was then incubated with different concentrations of r-thrombin (A), D419N-thrombin (B) and D419N-meizothrombin (C). Hirudin-bound protein was detected by affinity purified peroxidase-conjugated sheep anti-human thrombin immunoglobulin. Colour intensity was determined at 450 nm (absorbance 450 nm).

Fig. 2. Binding of r-thrombin, D419N-thrombin and D419N-meizothrombin to immobilized hirudin. Microtitteration plates coated with hirudin were incubated with different concentrations of r-thrombin (A), D419N-thrombin (B) and D419N-meizothrombin (C). Hirudin-bound protein was detected by affinity purified peroxidase-conjugated sheep anti-human thrombin immunoglobulin. Colour intensity was determined at 450 nm (absorbance 450 nm).
Fluorescence emission spectra of r-thrombin (A), D419N-thrombin (B) and D419N-meizothrombin (C) and of their complexes with hirudin. The protein concentration was 270 nM. Fluorescence emission spectra in the absence of hirudin (—) and in the presence of 1000 nM hirudin (...).

An addition of hirudin to r-thrombin, D419N-thrombin and D419N-meizothrombin resulted in a significant increase in fluorescence emission intensity at 341 nm.

The equilibrium fluorescence titration curves of 200 nM r-thrombin, D419N-thrombin and D419N-meizothrombin by hirudin (10–500 nM) are shown in Figure 4. Sharp breaks in the titration curves were obtained at the molar equivalence points for r-thrombin, D419N-thrombin and hirudin (Figure 4).

To test whether hirudin-induced increase in fluorescence emission results from structural changes in the γ-loop of thrombin, Trp468 was replaced by Phe in D419N-prothrombin to yield the double mutant D419N/W468F-prothrombin. D419N/W468F-prothrombin was expressed in CHO cells, converted into D419N/W468F-thrombin and affinity purified using immobilized hirudin C-terminal peptide (Figure 1D).

Fluorescence emission spectra of D419N/W468F-thrombin and of its complex with hirudin (Figure 5A) and the equilibrium fluorescence titration curve of the protein with hirudin (Figure 5B). No significant differences were observed between D419N/W468F-thrombin and D419N-thrombin.

r-Thrombin, D419N-thrombin and D419N-meizothrombin (200 nM) were incubated with up to 10 μM fibrinopeptide A. No change in fluorescence emission intensity was detected for any of the molecules. Incubation of 200 nM r-thrombin and D419N-thrombin with the C-terminal hirudin peptide 54–65 at the molar equivalence points for D419N-meizothrombin and hirudin (Figure 4c). The total fluorescence enhancement was 0.24. Similarly, the fluorescence titration curve did not reflect a simple binding mechanism of a single ligand binding at a single binding site with a constant association constant.
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Fig. 5. Effect of hirudin on D419N/W468F-thrombin. (A) Fluorescence emission spectra of D419N/W468F-thrombin and of its complexes with hirudin. The protein concentration was 270 nM. Fluorescence emission spectra in the absence of hirudin (—) and in the presence of 1000 nM hirudin (...). (B) Fluorescence titration of D419N/W468F-thrombin by hirudin. The protein concentration was 200 nM. Added hirudin concentration varied between 10 and 500 nM.

Fig. 6. Fluorescence titration of r-thrombin (A) and D419N-thrombin (B) by C-terminal hirudin peptide 54–64. The protein concentration was 200 nM. Added C-terminal hirudin peptide concentration varied between 10 and 500 nM.

resulted in a decrease in fluorescence emission intensity of less than 5% (Figure 6). However, at identical conditions the incubation of rD419N-meizothrombin with C-terminal hirudin peptide 54–65 did not change fluorescence intensity. Equivalent results were obtained with 200 nM r-thrombin, D419N-thrombin and D419N-meizothrombin and the C-terminal hirudin peptide 40–65 (not shown). Fluorescence emission intensity of r-thrombin and D419N-thrombin was reduced by less than 5% in the presence of 500 nM C-terminal hirudin peptide 40–65. Fluorescence emission of D419N-meizothrombin was not effected by the C-terminal hirudin peptide 40–65.

Discussion

Due to the replacement of Asp419 by Asn, D419N-thrombin and D419N-meizothrombin exhibited no clotting activity and titration resulted in no detection of the active catalytic centre. Both D419N-thrombin and D419N-meizothrombin exhibited no autocatalytic degradation activity (Fischer et al., 1996b). The structurally stability of D419N-thrombin and D419N-meizothrombin enabled the characterization of the binding to hirudin at equilibrium conditions.

Previous crystallographic analyses of the hirudin–thrombin complex have revealed that the N-terminal core domain of hirudin binds to thrombin with the three N-terminal residues protruding into the active site cleft. The C-terminal region of hirudin makes numerous contacts with regions remote from the catalytic centre, especially with the anion binding exosite (Rydel et al., 1990, 1991). The results in the present study revealed no qualitative differences in the interaction of hirudin with D419N-meizothrombin and D419N-thrombin. Binding to solid phase-immobilized hirudin in an ELISA demonstrated that D419N-meizothrombin exhibited hirudin binding activity similar to D419N-thrombin. Both D419N-meizothrombin and D419N-thrombin were affinity purified by an immobilized C-terminal hirudin peptide. D419N-meizothrombin and D419N-thrombin exhibited strong binding affinity to the immobilized C-terminal hirudin peptide and were eluted with 1.2 M KSCN. It is obvious that the anion binding exosite is active in D419N-meizothrombin and is accessible to hirudin as it is in D419N-thrombin. Binding of C-terminal hirudin peptides to D419N-meizothrombin did not change the intrinsic fluorescence emission. Less than 5% decrease of fluorescence intensity observed for binding of C-terminal hirudin peptides to D419N-thrombin demonstrated minor structural changes and may result from more flexibility of D419N-thrombin compared with D419N-meizothrombin, due to the loss of about half of the molecule (fragment 1 and 2) during conversion of D419N-prothrombin to D419N-thrombin. Fibrinopeptide A exhibited no effect on intrinsic fluorescence of D419N-meizothrombin and D419N-thrombin. Equilibrium fluorescence analysis showed that the interaction of hirudin with D419N-meizothrombin resulted in a significant increase in fluorescence emission at 341 nm. Similar results were obtained for D419N-thrombin. At equilibrium, maximum hirudin-induced fluorescence enhancement of D419N-meizothrombin and D419N-thrombin was 0.24 and 0.32, respectively. The total number of tryptophan residues in meizothrombin and

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thrombin is 14 and 9, respectively. At identical concentration and in the absence of hirudin, intrinsic fluorescence of D419N-mezothrombin was 1.7-fold higher than intrinsic fluorescence of D419N-thrombin. Assuming that the hirudin-mediated structural change in mezothrombin and thrombin is limited to the structural domains included in the thrombin molecule, equilibrium fluorescence titrations of D419N-mezothrombin and D419N-thrombin reflect identical structural events. Apparently, binding of hirudin via its C-terminal part to the anion binding exosite and via its N-terminal residues into the active site cleft resulted in major structural changes equally in D419N-mezothrombin and D419N-thrombin.

Previous kinetic studies have demonstrated that N- and C-terminal fragments of hirudin bind to thrombin with different rates (Stone and Hofsteenge, 1986; Jackman et al., 1992). Different binding and inhibitor constants were determined for N- and C-terminal fragments of hirudin binding to the active site cleft and to the anion binding exosite, respectively (Dennis et al., 1990; Schmitz et al., 1991). Hyperbolic and co-operative inhibitory effects of different hirudin peptides have been described in the past. It has been shown (Stone and Hofsteenge, 1986, 1990) that hirudin binds first via its C-terminal domain before proceeding to inhibit the thrombin active site. Kinetic and spectroscopic studies demonstrated that the binding of C-terminal hirudin peptides at the anion-binding exosite results in significant changes in the Michaelis constant for peptideyl p-nitrophenyl substrate, apparently due to some structural changes in the active site of thrombin (Konno et al., 1988; Mao et al., 1988; Dennis et al., 1990). These previous results fit well with our observations. Fluorescence titration of both D419N-thrombin and D419N-mezothrombin showed that hirudin binding-mediated structural changes do not reflect a simple association reaction, rather indicated a co-operative binding event. This made it virtually impossible to determine reliable binding constants. The majority of existing binding constants of thrombin and hirudin found in the literature are truly inhibitor constants and have been derived from kinetic inhibition experiments in which the thrombin-catalysed hydrolysis of substrate is inhibited by hirudin. However, this reflects only the local reaction at the active site cleft and at the catalytic centre and therefore cannot be interpreted as the thrombin-hirudin binding constant.

By comparing the three-dimensional structures of D-Phe-Pro-Arg-CH$_2$-thrombin and hirudin–thrombin complex (Rydell et al., 1990, 1991; Bode et al., 1992), tryptophan residues potentially influenced by the hirudin binding can be identified. Apparently, three of the nine tryptophan residues in the thrombin appeared to undergo changes in their environment. These residues are Trp370, Trp468 and Trp547. By far the most dramatic change involves Trp468 that is located in the γ-loop of thrombin. Evidence that the γ-loop undergoes a structural change comes from crystallographic studies (Grütter et al., 1990; Rydell et al., 1990, 1991; Bode et al., 1992), fluorescence spectroscopy studies (Jackman et al., 1992; Parry et al., 1993) and limited proteolysis experiments (Parry et al., 1993). To test whether Trp468 acts as probe for structural changes of thrombin in the hirudin–thrombin complex, we produced recombinant D419N/W468F-prothrombin. D419N/W468F-prothrombin was converted in vitro into D419N/W468F-thrombin and was affinity purified by immobilized C-terminal hirudin peptide. Surprisingly, fluorescence emission spectroscopy of D419N/W468-thrombin and of its complex with hirudin, as well as fluorescence titration with hirudin produced results similar to D419N-thrombin. Obviously, Trp468 does not reflect the hirudin-induced structural changes of thrombin. Thus, the conformation of the γ-loop in the crystal structure of D-Phe-Pro-ArgCH$_2$-thrombin (Bode et al., 1992) and the thrombin–hirudin complex (Grütter et al., 1990; Rydell et al., 1990, 1991) probably is different in solution. Structural analysis of thrombin obtained from different crystal forms recently demonstrated that crystal packing forces may contribute to different conformations of this loop (Priestle et al., 1993).

To obtain recombinant prothrombin-derived molecules which do not undergo autolysis during purification and equilibrium fluorescence studies, D419N-thrombin and D419N-mezothrombin were produced. For comparison r-thrombin was prepared. While at identical concentration the intrinsic fluorescence emission was similar for r-thrombin and D419N-thrombin, hirudin-induced fluorescence change was more dramatic in D419N-thrombin compared with r-thrombin. This result suggests that the replacement of Asp149 by Asn in the catalytic centre of thrombin favoured the structural change induced by hirudin binding. In the catalytic centre of serine proteases, histidine is poised to accept the proton from the serine hydroxyl group when this oxygen atom carries out a nucleophilic attack on the substrate. The role of the carboxyl group of aspartate in the catalytic triad is to stabilize the positively charged form of histidine in the transition state. In addition, aspartate orients histidine and ensures it is in the appropriate tautomeric form to accept a proton from serine (Antonov, 1993). Craik et al. (1987) and Sprang et al. (1987) have analysed the function and three-dimensional structure of a trypsin mutant with catalytic site aspartic acid replaced by asparagine. In the mutant, asparagine is a hydrogen bond donor to histidine. This hydrogen arrangement prevents histidine from accepting a hydrogen bond from serine and results in a loss of nucleophilicity of serine. Due to the structural homology of the catalytic centre, it is very likely that the identical structural situation applies to D419N-thrombin. Replacement in thrombin of active-site Asp149 by Asn results in a loss of polarity in the catalytic centre. In the hirudin–thrombin complex, the N-terminal domain of hirudin binds at the active-site of thrombin where the side-chains of Ile1 and Tyr3 of hirudin occupy the apolar binding-site by making numerous hydrophobic contacts including hydrophobic interactions with side-chains of Trp370 and Trp547 (Rydell et al., 1990, 1991). Based on our results it can be speculated that the loss of polarity in the catalytic centre of D419N-thrombin enables a reinforcement of hydrophobic contacts and more pronounced decrease in solvent exposure and, thus may lead to the fluorescence enhancement observed.

In a recent model for the specificity of fibrinogen cleavage by thrombin (Stubbs and Bode, 1993), fibrinogen Aα-chain amino acids Asp7 to Arg16, contained in the fibrinopeptide A, make numerous contacts to the catalytic cleft and the apolar binding pocket of the thrombin molecule. Cleavage at position Arg16–Gly17 releases fibrinopeptide A. However, the thrombin cleavage of fibrinogen requires more than fibrinopeptide recognition. Stubbs and Bode (1993) suggested that the residues Phe35 to Tyr43 of the fibrinogen A-chain bind to the anion binding exosite of thrombin which would result in most effective orientation of the entire fibrinogen Aα-chain at the thrombin surface prior cleavage. Hydrolysis at equivalent rate of peptidyl substrates by thrombin and mezothrombin demonstrate similar structures of the catalytic cleft and the active site. The results of the present study showed that
anion binding exosites are active in both proteins. Thus, the inability of meizothrombin to cleave fibrinogen results most likely from steric hindrance, probably by the F1/F2-peptide which by its size of 271 amino acids (about half of the prothrombin molecule) blocks the simultaneous binding the fibrinogen molecule (a covalently linked dimer of three peptide chains with a molecular weight of 340 000) at the catalytic site and the anion binding exosite.

In summary, our results indicated no qualitative differences between the binding of hirudin to thrombin and meizothrombin. Both molecules exhibit anion binding exosites and catalytic cleft binding sites which are similarly active in hirudin binding. Hirudin binding induces similar structural changes in thrombin and meizothrombin; however, not in the flexible γ-loop of the protein. Due to the binding of hirudin at the catalytic cleft and at the anion binding exosite the hirudin binding-induced structural change reflected a co-operative event. Since both the anion binding exosite and the catalytic cleft are active and simultaneously accessible, inability for fibrinogen cleavage by meizothrombin most likely results from steric blocking of fibrinogen by the F1/F2-peptide of meizothrombin.

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References

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