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Evidence for multiple enzyme site involvement in the modulation of thrombin activity by products of prothrombin proteolysis

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Abstract

Kinetic evidence is presented for the interaction of prothrombin with several distinctive topological regions of the thrombin molecule. Modulations of thrombin catalytic activity on the protein substrates prothrombin and prethrombin 1 are demonstrated that involve the fragment 1 and fragment 2 portions. The inhibitory effects are demonstrably non-competitive. In addition to exhibiting non-competitive inhibition, fragment 2 is capable of enhancing proteolysis by thrombin; and therefore to react with a second region of the enzyme. On the basis of the crystallographic studies of the complex between fragment 2 and thrombin (Arni et al., Biochemistry 32 (1992) 4727), this activating site is proposed to be associated with exosite II. The allosteric switch between procoagulant and anticoagulant activities identified from studies by Di Cera (Dang et al., Proc. Natl. Acad. Sci USA 92 (1995) 5977) could be 'thrown' by a macromolecular effector that is generated during thrombin formation — a plausible mechanism for switching that deserves further investigation. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Thrombin modulation; Fragment 1; Fragment 2; Thrombin inhibition; Blood coagulation; Proteolysis

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Abbreviations: F1, prothrombin fragment 1; F2-$A_{(1-13)}$, prothrombin fragment 2 formed by thrombin action on human prothrombin; gla, γ-carboxyglutamic acid

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1. Introduction

Interactions of thrombin with macromolecular effectors regulate the relative extents of proteolysis undergone by its numerous potential substrates in order to achieve the appropriate catalytic function. Molecular interpretation of these interactions that modulate the specificity of this key enzyme in blood coagulation has been facilitated greatly by the elucidation of the three-dimensional structure of α-thrombin and its complex with hirudin [1]. As well as defining the topography of the active site of α-thrombin, those crystallographic studies have identified several distinctive surface regions, or exosites, that are believed to play pivotal roles in the facilitation and control of proteolysis by thrombin. For example, there is the fibrinogen-binding region (exosite I), which also contributes to the interactions responsible for the inhibition of thrombin by hirudin. Another distinctive surface feature of α-thrombin is exosite II, the putative heparin-binding site for interaction with polyanions [2,3] and the F2-$A_{(1-13)}$ fragment of prothrombin [4].

The X-ray crystallographic demonstration that the interaction of thrombin with several macromolecular effectors or peptides derived from them entails occupancy of the fibrinogen-binding exosite [1,5] raises the possibility of there being more than one distinctive topological region involved in the modulation of thrombin by other macromolecular effectors. The multiplicity of thrombin function in hemostasis begs mechanisms by which temporal (e.g. fibrinogen clotting and Protein C activation) and spatial (e.g. binding to cellular receptors) specificity can be achieved [6]. The separate observations that thrombomodulin accelerates both thrombin activation of Protein C and thrombin inactivation by antithrombin III [7–9], whilst being competitive with hirudin binding [10–12], would certainly be consistent with that suggestion; and so would the observation that the binding of a heparin cofactor II-derived peptide accelerates thrombin inactivation by heparin cofactor II [13]. In that regard the demonstrated binding of bothrajaracin to exosites I and II [14] provides tangible experimental evidence of the need to probe further this concept that the modulation of thrombin activity by a single macromolecular effector may require molecular interpretation in terms of interaction with more than one distinctive topological region of the enzyme.

Our selection of prothrombin for further study stems from observations that prothrombin fragment 2 enhances the thrombin-catalyzed hydrolysis of TAME [15], whereas it inhibits the inactivation of thrombin by antithrombin III [16] and competes with thrombomodulin in Protein C activation [17]. These findings for a prothrombin-derived polypeptide point to the possibility that prothrombin, a substrate for thrombin, might also act as a regulator of thrombin function. As a substrate prothrombin seemingly exhibits classical Michaelis–Menten behavior [18]; and inhibits the thrombin-catalyzed hydrolysis of a chromogenic substrate [19]. However, inability to provide a rational explanation of several anomalies in our unpublished findings has prompted this more detailed investigation into the interactions of thrombin with prothrombin and the two resulting proteolysis products, fragment 1 and fragment 2.

2. Methods

2.1. Materials

The prothrombin, antithrombin III and α-thrombin were from batches used previously [20–22]. Enzyme concentrations were determined by active-site titration [23], whereas prothrombin concentrations were estimated spectrophotometrically at 280 nm [24]. Previous procedures [25,26] were used to prepare prethrombin 1 and prothrombin fragments F1 and F2-$A_{(1-13)}$, the gla and kringle regions of the latter being also isolated [26]. $Hirudin_{(54-65)}$ was from Sigma.

2.2. Kinetic studies of prothrombin and prethrombin 1 hydrolysis

The kinetics of thrombin-catalyzed proteolysis of prothrombin and prethrombin 1 were followed by means of fixed-time incubations at 25°C of enzyme-substrate-modifier mixtures in Hepes–chloride buffer (0.05 M Hepes/HCl–0.125 M

NaCl), pH 7.4, I 0.15, supplemented with PEG6000 (1 mg/ml) to minimize protein losses by absorption. The reaction was terminated by addition of D-Phe–Pro–Arg–chloromethylketone. In experiments where $CaCl_2$ (10 mM) was included in reaction mixtures the NaCl concentration was decreased to 0.095 M to maintain constant ionic strength. Proteolytic reaction products were separated [15,16] by HPLC on a MonoQ (HR 5/5) column (Pharmacia) at a flow rate of 1.0 ml/min at pH 7.5 with 0.05 M Tris–HCl, 1 mg/ml PEG 6000 buffer plus 0.1 M NaCl (A); plus 2.0 M NaCl (B); plus 0.1 M NaCl, 0.2 M $CaCl_2$ (C). Elution employed intervals (0–2.0 min), 100% A; (2.1–6.0 min), 92% A, 8% B; (6.1–13.0 min), 100% A to 90% A, 9% B, 1% C; (13.1–21.0 min), 90% A, 9% B, 1% C to 80% A, 10% B, 10% C; (21.1–25 min), 100% B. The sample (0.5 ml, 0.05–10 μM product or reactant) was applied to the column equilibrated with A. Quantification of all reactants and products was by integration of peak areas in the spectrophotometric record (280 nm) of the elution profile. Initial velocities were calculated from best-fit descriptions of the progress curves in terms of the integrated rate equation for a first-order reaction [27,28].

2.3. Alternative substrate assays with a chromogenic substrate

In studies based on chromogenic substrate assays the initial velocity of release of *p*-nitroalanine from CH_3SO_2-D-Leu–Gly–Arg–*p*-nitroanilide (CBS 31.39, American Bioproducts and Diagnostica Stago) was measured by following the increase in absorbance at 405 nm with a Cary 219 recording spectrophotometer. Concentrations of chromogenic substrate were determined spectrophotometrically at 342 nm on the basis of an absorption coefficient of 8210 M^{-1} cm^{-1} [29]. For experiments with prothrombin (0–57 μM) and fibrinogen (0–4.5 μM) the concentrations of thrombin and CBS 31.39 were 5 nM and 180 μM, respectively. Polymerization of the fibrin produced in the reactions with fibrinogen was prevented by including 6 mM Gly–Pro–Arg–Pro (Diosynth, BV and Sigma), which has no detectable effect on the thrombin-catalyzed release of fibrinopeptide A [30] or on thrombin-catalyzed hydrolysis of CBS 31.39. The kinetic parameters for hydrolysis of CBS 31.39 were obtained by least-squares analysis of full progress curves with allowance for competitive product inhibition [31]: values of 226 ± 5 μM for K_m, 253 ± 7 μM for K_I and 45.7 ± 0.5 s^{-1} for k_c were obtained.

Alternative substrate kinetic data were fit by least-squares regression to the standard expression for competitive inhibition, namely

$$v_i = k_c[E]_t[S]_o/\{[S]_o + K_m(1 + [I]_o/K_I)\} \qquad (1)$$

where v_i is the initial velocity in an experiment with total thrombin concentration $[E]_t$ and initial CBS 31.39 concentration $[S]_o$: K_m is the Michaelis constant for chromogenic substrate and K_I that for the competing (inhibitory) substrate, present at initial concentration $[I]_o$. Results were fit to Eq. (1) with K_I as the parameter to be determined.

2.4. Thrombin inactivation by antithrombin III

Apparent second-order rate constants for the inactivation of thrombin by antithrombin III were determined by monitoring proteinase inactivation through chromogenic substrate hydrolysis [32]. The concentration of antithrombin III was more than 500-fold greater than that of thrombin in these experiments.

3. Results

3.1. Kinetics of prothrombin cleavage by thrombin

Evidence that prothrombin can act as an effector of its own catalytic cleavage by thrombin is presented in Fig. 1, which summarizes the dependence of the initial velocity of Arg_{155}–Ser_{156} scission upon human prothrombin concentration in the absence of Ca^{2+}. For low prothrombin concentrations the reaction seemingly conforms with Michaelis–Menten behavior in the sense that the normalized initial velocity ($v_i/[E]_t$) increases with substrate concentration (the interpretation adopted by Silverberg [18] for analysis of data for

bovine prothrombin over a restricted concentration range). However, extension of the range of prothrombin concentration reveals insufficiency of that interpretation, which presumes the existence of a monotonic increase in $v_i/[E]_t$ to an asymptotic value reflecting the kinetic behavior of substrate-saturated enzyme. The simplest kinetic scheme which would account for the existence of the observed maximum in the experimental dependence (Fig. 1) is a hyperbolic mixed inhibition, a mechanism generally conceived as involving a second site in addition to the active site on the enzyme [33]. In the sense that prothrombin seems to be acting as both substrate and inhibitor, a second interaction with an inhibitory site on thrombin is implicated.

Additional evidence for prothrombin as a potential modulator of thrombin activity comes from studies of its effect on the thrombin-catalyzed hydrolysis of the chromogenic substrate CBS 31.39. Consideration of the interaction between thrombin and prothrombin to be restricted to proteolytic function of the enzyme leads to the conclusion that the alternative substrate should act as a classical competitive inhibitor of chromogenic substrate hydrolysis. This effect is certainly observed with fibrinogen as the alternative substrate under conditions where proteolysis of the peptide bond at Arg16 of the α-chain is the sole reaction in competition with hydrolysis of CBS 31.39 hydrolysis (Fig. 2A). Interpretation of these results in terms of Eq. (1) leads to a K_I of 8.1 ± 0.5 μM for the inhibition by the competing substrate (a value consistent with published estimates of K_m (3 ± 18 μM) for the release of fibrinopeptide A from fibrinogen [32,34,35]). Such behavior contrasts markedly with results from experiments with prothrombin as the alternative substrate (Fig. 2B). Whereas pronounced inhibition is predicted on the basis of a K_m of 6 μM, the value inferred from the low-concentration portion of Fig. 1, the experimental results signify very little, if any, effect of prothrombin concen-

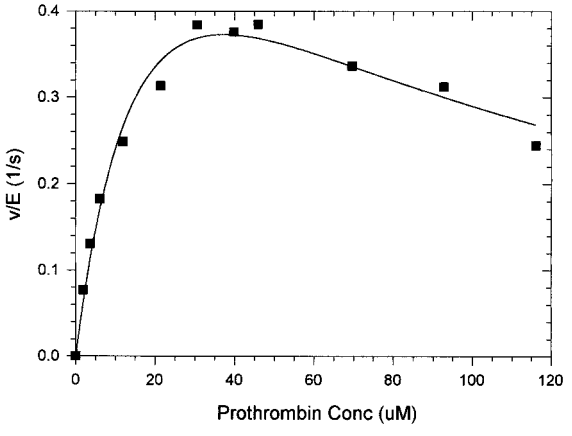

Fig. 1. Dependence of the normalized initial velocity (v divided by total enzyme concentration) of Arg_{155}–Ser_{156} scission upon substrate concentration in the thrombin-catalyzed cleavage of prothrombin at 25°C in Hepes–chloride buffer, pH 7.4, I 0.15. The solid line is calculated from the 'classical' substrate inhibition model [33], with values for: $K_S = 100$ μM, $\alpha = 10$ ($K_I = \alpha K_S$), $k_c = 3.15$ s^{-1} and $\beta = 0.3$.

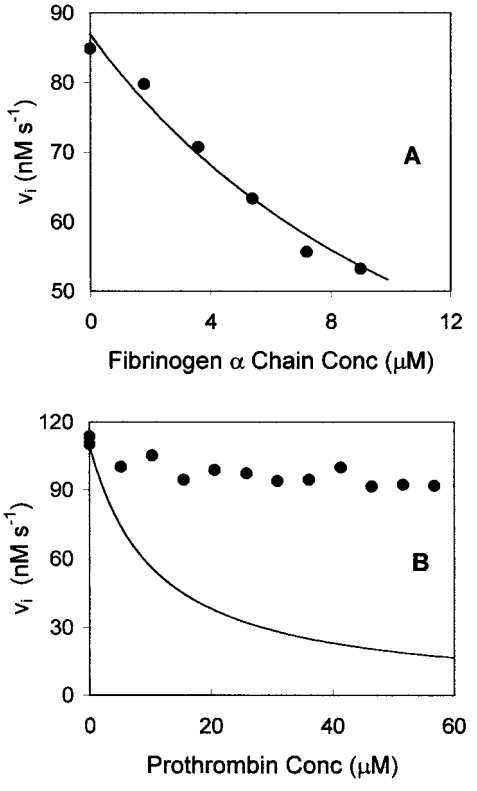

Fig. 2. Studies of the interactions of (A) fibrinogen and (B) prothrombin with thrombin by means of chromogenic assays with 180 μM CBS 31.39. The solid line in (A) is the best-fit relationship in terms of Eq. (1), i.e. for a K_m of 8.1 μM. The solid line in (B) is that predicted by Eq. (1) and a K_I of 6 μM (the value inferred from the initial portion of Fig. 1).

tration on CBS 31.39 hydrolysis. Clearly, these results are also incompatible with interpretation solely from a prothrombin–thrombin interaction that is restricted to the active-site region of the enzyme.

From the viewpoint of establishing more definitively the possible existence of multiple sites on thrombin for interaction with a given macromolecular effector, the prothrombin–thrombin system is complicated by the fact that catalytic cleavage occurs at Arg_{155}–Ser_{156} and Arg_{284}–Thr_{285} to form the respective F1 and F2-$A_{(1-13)}$ prothrombin fragments, which also have the potential to act as macromolecular effectors. To simplify the model system we have therefore eliminated the problem of double proteolytic scission by using prethrombin 1 (prothrombin with the F1 fragment removed) as substrate. F1 and F2-$A_{(1-13)}$, the two activation fragments derived from prothrombin, have been selected as models for the regions of the prothrombin from which they are derived.

3.2. Kinetics of prethrombin 1 cleavage

Kinetic parameters for the thrombin-catalyzed hydrolysis of the single Arg–Thr peptide bond in prethrombin 1 have been determined by analyzing progress curves (Fig. 3). For this substrate the concentration employed (usually 3 μM) was sufficiently low in relation to the Michaelis constant (K_m) for the progress curves to be described by first-order rather than Michaelis–Menten kinetics. In Michaelis–Menten terms the pseudo-first-order rate constant (k_{obs}) so obtained defines $(k_c/K_m)[E]_t$, the product of total enzyme concentration ($[E]_t$) and a second-order rate constant defined by the ratio of the catalytic rate constant (k_c) to the Michaelis constant [27]. Progress curves for the thrombin-catalyzed disappearance of prethrombin 1 and appearance of F2-$A_{(1-13)}$ are described adequately by a single kinetic rate constant. Furthermore, the inset to Fig. 3 establishes the linearity of the dependence of the pseudo-first-order kinetic constant upon substrate concentration, thereby verifying the approximation that $[S] \ll K_m$ and hence identification of k_{obs} as $(k_c/K_m)[E]_t$.

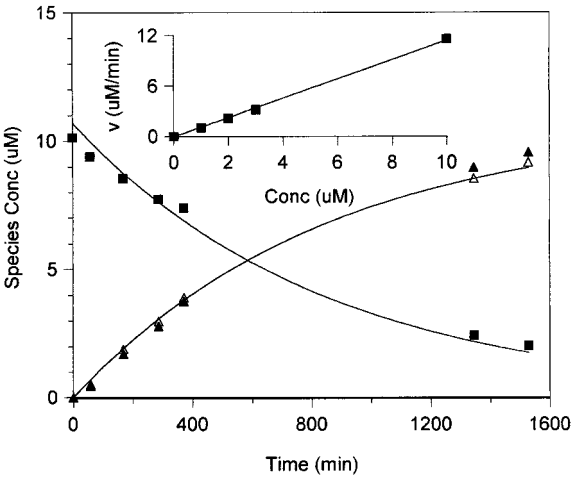

Fig. 3. Progress curves for the thrombin-catalyzed cleavage of prethrombin 1 at 25°C in Hepes–chloride buffer, pH 7.4, I 0.15. Time courses of prethrombin 1 disappearance (□) and product formation (△,▲) for the hydrolysis of 10 μM prethrombin 1 by 0.2 μM thrombin: the solid lines are calculated from global curve-fitting of the results in terms of a single pseudo-first-order rate constant, viz. 19.7×10^6 s^{-1}. Inset: dependence of the initial velocity (v) upon prethrombin 1 concentration.

3.3. Effect of F2-$A_{(1-13)}$ on proteolytic cleavage of prethrombin 1

The effect of F2-$A_{(1-13)}$ on k_{obs} for proteolytic cleavage of the single peptide bond in human prethrombin 1 that is susceptible to thrombin is summarized in Fig. 4, which refers to experiments with 0.2 μM thrombin, 3 μM prethrombin 1, and a range of F2-$A_{(1-13)}$ concentrations, expressed on a logarithmic scale in the inset to Fig. 4. The first point to note in relation to Fig. 4 is the essential identity of results obtained for reaction mixtures supplemented with 1 mM EDTA and 10 mM Ca^{2+}, which is not surprising in the sense that the Gla domain (the source of dominant metal-ion effects in prothrombin) is not present in the F2-$A_{(1-13)}$ fragment. Secondly, the form of the curve in Fig. 4 signifies that the F2-$A_{(1-13)}$ segment of prothrombin acts both as an activator and as an inhibitor of prethrombin 1 proteolysis by thrombin. Furthermore, use of this fragment as macromolecular effector (modifier) rather than the whole prothrombin molecule unambiguously

shows the inhibition to be non-competitive, because modifier-saturated thrombin is still able to catalyze proteolytic cleavage of prethrombin 1, albeit at a decreased rate; i.e. the limiting value of k_{obs} at high concentrations of F2-$A_{(1-13)}$ is non-zero. The first important conclusion to emerge from the non-competitive nature of the behavior shown in Fig. 4 is that neither the activatory nor inhibitory effects of F2-$A_{(1-13)}$ result from interactions with the active-site region of thrombin. Qualitative reasoning based on the inset of Fig. 4 signifies that the effector interaction giving rise to activation must be governed by a dissociation constant which may be as large as 10 μM, whereas that associated with inhibition is less than 100 μM. Because a peak rather than a plateau is observed in Fig. 4, the thrombin exosites that are responsible for the two phenomena must exhibit affinities for F2-$A_{(1-13)}$ that are of the same order of magnitude. Secondly, we note that the consequences of the inhibitory interaction appear to outweigh those of the activatory phenomenon, because the fully saturated thrombin-modifier complex exhibits only 40% of the catalytic capability of unmodified enzyme. Despite significant differences, the general similarity between the F2 kringle from prothrombin and the lysine-binding kringles of plasminogen [4] prompted an investigation of the effect of lysine on the activation by F2 of the cleavage of Arg_{284}–Thr_{285} in prethrombin 1. The extent of activation was increased slightly in the absence of Ca^{2+} at 10 mM lysine; no effect was seen in the presence of 10 mM Ca^{2+} (data not shown).

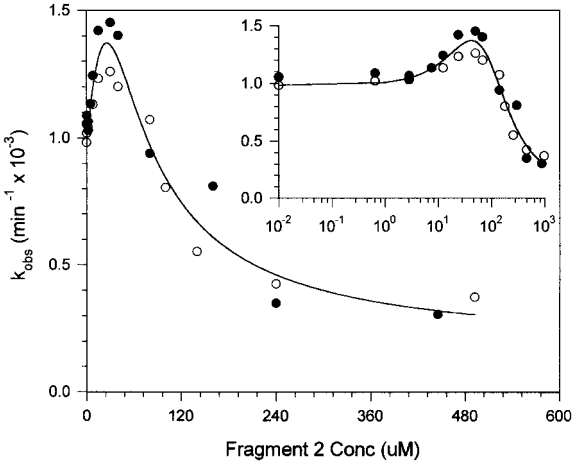

Fig. 4. Modulation of thrombin-catalyzed hydrolysis of 3 μM prethrombin 1 (pH 7.4, I 0.15, 25°C) by F2-$A_{(1-13)}$ in the absence (○) and presence (●) of Ca^{2+}, the enzyme concentration being fixed at 0.2 μM. Inset: the same dependence with effector concentration expressed on a logarithmic scale. The solid lines are calculated from the best fit parameters to Eq. (3b).

3.4. Effect of F2-$A_{(1-13)}$ on thrombin inactivation by antithrombin III

Use of the hydrolysis of the chromogenic substrate CBS 31.39 to monitor the effect of F2-$A_{(1-13)}$ on thrombin inactivation by antithrombin is summarized in Fig. 5. Despite the relative sparsity of data, they suffice to establish that the effect of fragment 2 on the inhibition of thrombin inactivation by antithrombin III mimics the corresponding effect on prethrombin 1 proteolysis by thrombin: the modulation is again both activatory and non-competitively inhibitory. In that regard similar findings were reported several years ago for the corresponding bovine system. Summarized in the inset to Fig. 5 are results from Table 1 of Walker and Esmon [16] for the same reaction studied by means of the fibrinogen clotting assay. Although no comment was made at the time, these results also signify a role for F2 as both an activatory and an inhibitory modulator of bovine thrombin inactivation by antithrombin III.

3.5. Effect of F1 on proteolytic cleavage of prethrombin 1

From the kinetic results presented in Fig. 6A for F1 in the presence of EDTA it is evident that this human prothrombin fragment is also a macromolecular effector of prethrombin 1 cleavage by thrombin. In this instance, however, the modulation is confined to non-competitive inhibition, there being no activation observed with this kringle. Upon introduction of more rigid and compact structure into the F1 by the inclusion of Ca^{2+} [36,37], F1 ceases to be a macromolecular inhibitor of prethrombin 1 cleavage by thrombin.

Non-competitive inhibition similar to that seen

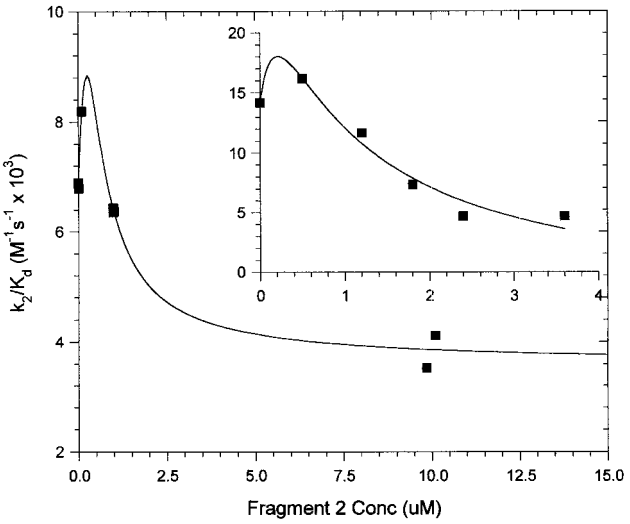

Fig. 5. Effect of F2-$A_{(1-13)}$ on the inactivation of human thrombin by antithrombin III. Inset: results reported in Table 1 of Ref. [16]. The solid lines are calculated from the best fit parameters to Eq. (3b).

with F2-$A_{(1-13)}$ is observed for the kringle of F1 (Fig. 6B). The finding that the form of the concentration-dependence of k_{obs} for the Gla-less F1 kringle is essentially unchanged by the presence of Ca^{2+} could reasonably have been predicted. Ca^{2+} binds most extensively to the Gla domain

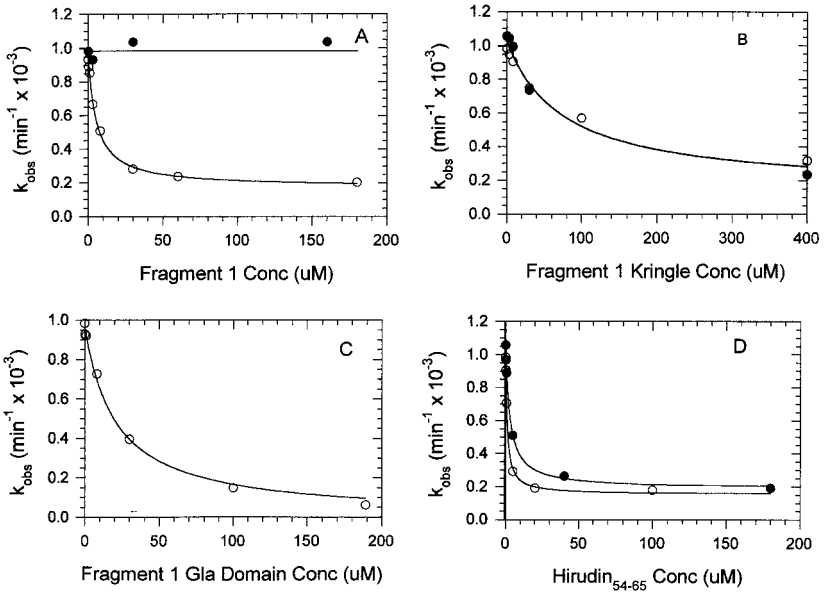

Fig. 6. Modifier effects of F1 and derivatives thereof on the cleavage of prethrombin 1 by thrombin in the absence (open symbols) and presence (closed symbols) of Ca^{2+}. (A) Inhibitory effect of F1. (B) Modulation by the F1 kringle (Gla-domainless F1). (C) Inhibition by the Gla domain of F1. (D) Corresponding effects of hirudin$_{(54-65)}$. The solid lines are calculated from the best fit parameters to Eq. (2), except for (A) in the presence of Ca^{2+} which is a linear regression fit to a straight line.

[38] and at most to one site on the kringle of F1 [39]. In the absence of Ca^{2+} the Gla domain of F1 inhibits the action of thrombin on prethrombin 1 in a seemingly competitive manner (Fig. 6C). However, in view of the non-competitive nature of the inhibition exhibited by all other F1-related systems, it seems likely that the Gla domain in the absence of Ca^{2+} might also be binding to the same exosite as F1 in the absence of Ca^{2+} (but in a manner that obstructs the approach of the macromolecular prethrombin 1 substrate to the active-site cleft). It must be noted that, although the kringle of F1 inhibits thrombin non-competitively in both the presence and absence of Ca^{2+} (Fig. 6B), the fact that the whole F1 fragment is inhibitory only in the absence of metal ion (Fig. 6A) renders this observation of in vitro mechanistic significance only. The experiments in the absence of Ca^{2+} thus merely provide a reference point for interpretation of the effects of Ca^{2+}, rather than support for the existence of the modulatory sites that give rise to the activation and inhibition that occupancy of these sites produces.

A quantitative comparison of the inhibitory effects of F1 and fragments derived therefrom is presented in Table 1. The calculated equilibrium constants summarize the analyses of the results (Fig. 6A–C) in terms of the simplest mechanistic scheme for such inhibition, namely,

$$\begin{array}{ccccc} E + S & \overset{(K_m)_E}{\rightleftarrows} & ES & \overset{(k_c)_E}{\rightarrow} & E + \text{products} \\ + & & & & \\ M & & & & \\ K_{EM} \uparrow\downarrow & & & & \\ EM + S & \overset{(K_m)_{EM}}{\rightleftarrows} & EMS & \overset{(k_c)_{EM}}{\rightarrow} & EM + \text{products} \end{array}$$

where $(K_m)_E$ and $(k_c)_E$ are the two Michaelis–Menten parameters for catalytic cleavage of prethrombin 1 (S) by thrombin (E). K_{EM} is the dissociation constant describing the interaction between macromolecular modifier (M) and an exosite on thrombin to form a complex EM for which the Michaelis–Menten parameters are $(K_m)_{EM}$ and $(k_c)_{EM}$. Because of the low substrate concentration relative to K_m in all these experiments, the reaction ES + M ⇄ ESM is omitted to simplify further the analysis. After allowance for the fact that the combined concentrations of enzyme–substrate complexes are contributing negligibly to the total concentration of enzyme (the condition for pseudo-first-order kinetics to apply), classical consideration of this reaction scheme leads to the conclusion that the rate constants measured in the presence of modifier, $(k_{obs})_M$, are given by the expression

$$(k_{obs})_M = \frac{\{(k_c/K_m)_E + (k_c/K_m)_{EM}[M]/K_{EM}\}[E]_t}{1 + [M]/K_{EM}} \quad (2)$$

Non-linear regression analysis of the results in terms of Eq. (2) thus leads to the values of the dissociation constant for the interaction of modifier with enzyme (K_{EM}), and also the ratio of Michaelis–Menten parameters, $(k_c/K_m)_{EM}/(k_c/K_m)_E$, for effector-saturated and effector-free enzyme given in Table 1.

Table 1
Inhibitory characteristics of various modifiers on the thrombin-catalyzed proteolysis of human prethrombin 1 (pH 7.4, *I* 0.15, 25°C)

Effector	K_{EM} (μM)[a]	$(k_c/K_m)_{EM}/(k_c/K_m)_E$[a]
Fragment 1[b]	5.3 (±0.5)	0.18 (±0.02)
F1 kringle[b,c]	75 (±20)	0.14 (±0.07)
F1 Gla domain[b]	21 (±2)	0[d]
Hirudin peptide[b]	1.1 (±0.1)	0.16 (±0.01)
Hirudin peptide[c]	3.1 (±0.5)	0.19 (±0.03)

[a] Calculated by curve-fitting in terms of Eq. (2).
[b] In the absence of Ca^{2+} (presence of 1 mM EDTA).
[c] In the presence of 10 mM Ca^{2+}.
[d] Kinetics symptomatic of competitive inhibition (see text).

3.6. Inhibition of proteolysis by hirudin$_{(54-65)}$ fragment

A logical next step of this investigation is to compare the finding that the inhibitory actions of F2-A$_{(1-13)}$, F1, and the various F1 segments are non-competitive with the corresponding effects of C-terminal hirudin peptides, which are known to

interact with the fibrinogen-binding exosite [5,10−12,40,41]. The effects of hirudin$_{(54-65)}$ fragment on the thrombin-catalyzed proteolysis of prethrombin 1 in the presence and absence of Ca^{2+} are summarized in Fig. 6D. It is evident that the inhibition is again non-competitive with the effect of Ca^{2+} slight, but significant (Table 1). Because of the similarity between these effects and those observed for prethrombin 1 with F1 and F1 kringle (Table 1), and also those for the inhibitory effect of F2-A$_{(1-13)}$, (Table 1), we suggest that the inhibitory actions of these macromolecular effectors results from interaction with the fibrinogen-binding exosite of thrombin on the grounds that it is the site to which the C-terminal hirudin peptides bind [5,10−12,40,41]. Indeed, competition between F2-A$_{(1-13)}$ and hirudin fragments has been reported recently [42].

3.7. Effects of fragments 1 and 2 on hydrolysis of CH_3SO_2-D-Leu−Gly−Arg−p-nitroanilide (CBS 31.39)

The effects of fragments 1 and 2 on the hydrolysis of CH_3SO_2-D-Leu−Gly−Arg−*p*-nitroanilide (CBS 31.39) were measured from the initial velocity of *p*-nitroaniline formation. Effects of F2-A$_{(1-13)}$ and F1 on the hydrolysis of the chromogenic substrate, CBS 31.39 are on both K_m and k_c, Fig. 7. The initial activating effect of F2-A$_{(1-13)}$ is primarily due to a greater relative decrease in K_m, than that in k_c, which thus leads to a net increase k_c/K_m. This is followed at higher F2-A$_{(1-13)}$ concentration by inhibition, an effect similar to that seen with prethrombin 1 as the substrate. Also in keeping with the findings for prethrombin 1 is the observation that F1 is only inhibitory, the decrease in k_c/K_m arising from a large increase in K_m and a small decrease in k_c.

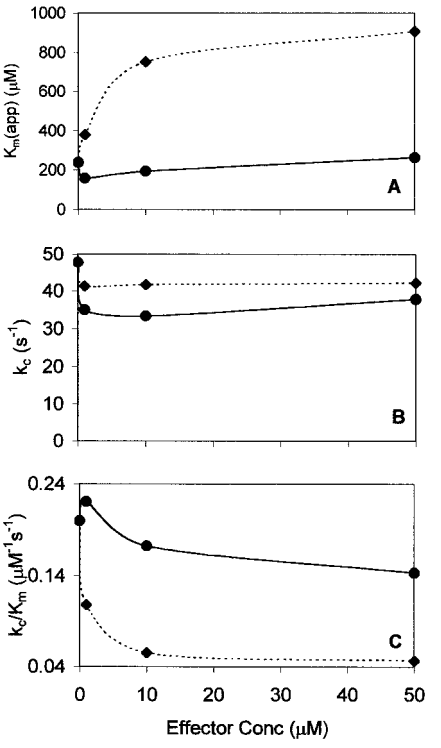

Fig. 7. Effect of F2-A$_{(1-13)}$ (●) and F1 (♦) on the thrombin-catalyzed hydrolysis of chromogenic substrate CBS 31.39 (pH 7.4, *I* 0.15, 25°C). Dependence of (A) the Michaelis constant K_m, (B) the catalytic rate constant k_c and (C) the second-order rate constant k_c/K_m upon effector concentration. The solid lines are smoothed lines produced by Microsoft Excel owing to the limited number of points relative to the number of parameters in Eq. (3b).

4. Discussion

The most significant finding in these studies is that there can be activation as well as non-competitive inhibition of thrombin when F2-A$_{(1-13)}$ is the macromolecular effector (Fig. 4). The observation of both activation and inhibition by F2 implies that this fragment must interact with at least two regions on the thrombin surface. This point has been noted in relation to a demonstration that adenine nucleotides are both activators and inhibitors of thrombin [43]. Failure to detect competition between F2 and fluorescently labeled hirugen, an exosite I-binding effector, has led to the conclusion that hirugen and F2 bind to different sites on thrombin [44]. X-ray crystallographic studies have demonstrated F2-A$_{(1-13)}$ interaction with the putative heparin-binding exosite (exosite II) of thrombin [4]. Measurements of direct binding of F2 to thrombin labeled with different, active site-directed fluorescence probes lead to equilibrium constants of the same magnitude, 3−22 μM [45], as those inferred from the kinetic studies reported here. The peptide, TR

52–69 from the thrombin receptor protein also binds to thrombin with a similar affinity, ~37 μM, at exosite I [46]. Interestingly, in the fluorescence probe binding studies [45], F2-$A_{(1-13)}$ can produce both enhancement and quenching of the fluorescence which depends on the structure of the probe and the peptide link to the active site His of thrombin. These observations are suggestive of subtle, but potentially important discrimination among the consequences of effector binding for the specificity of thrombin.

The fact that the F2-$A_{(1-13)}$ portion of prothrombin is an activator of thrombin activity raises the possibility that the initial phase of the velocity versus substrate behavior (Fig. 1) with prothrombin as both substrate and macromolecular effector may reflect activation at the lower prothrombin concentrations as well as an increase in velocity as the result of an increased extent of active-site saturation with substrate. The subsequent decrease in reaction velocity with substrate concentration may be related to the inhibition that is attributed to interaction with the second exosite. It now seems likely that interaction of prothrombin with thrombin involves three distinct regions of the thrombin molecule: (1) interaction with the active site in the role of prothrombin as a substrate, (2) interaction with one exosite in its role as an activator, and (3) interaction with the other exosite as a non-competitive inhibitor.

The effects of F2-$A_{(1-13)}$ and F1 on the hydrolysis of the chromogenic substrate, CBS 31.39 demonstrated here are on both K_m and k_c (Fig. 7). Activation by F2-$A_{(1-13)}$ is primarily due to a decrease in K_m and thus an increase in k_c/K_m which is followed at higher F2-$A_{(1-13)}$ concentration by inhibition. As noted above, the inhibitory effect of F1 (also seen with prethrombin 1 as substrate) arises predominantly from an increase in K_m for CBS 31.39. The pattern of activation and inhibition by F2-$A_{(1-13)}$ and inhibition by F1 is thus the same as that exhibited by these fragments on the cleavage of prethrombin 1. In contrast to the observations made here with CBS 31.39, the earliest report of an effect of F2 on a synthetic substrate, Tos–Arg-OMe [15], showed only activation. This observation, which was made under conditions of low ionic strength and in the absence of Na^+, is consistent with the dominantly electrostatic interaction between F2-$A_{(1-13)}$ and exosite II [4]. The absence of Na^+ might indicate that activation as seen with this particular substrate is in some way reflective of the conformation of thrombin described as the 'anticoagulant form' [47]. Thrombin exhibits preferential action on Protein C, rather than fibrinogen under conditions similar to those in Ref. [15]. When prothrombin, rather than the individual fragments is investigated for its effect on the hydrolysis of CBS 31.39, the effect observed is small, Fig. 2B. It is tempting to rationalize this observation on the basis of the opposing effects of F1 and F2-$A_{(1-13)}$, inhibitory effects only by F1 and activation and inhibition by F2-$A_{(1-13)}$. Only additional investigation will clarify the complex nature of the modulation by the fragments of prothrombin and the other proteins and peptides that alter thrombin catalytic properties.

The simplest, two-site mechanistic scheme that can account for both the activation and non-competitive inhibition observed with F2-$A_{(1-13)}$ as the macromolecular effector of prethrombin 1 cleavage by thrombin is the following.

$$
\begin{array}{ccc}
 & \mathrm{EM} + \mathrm{S} \overset{(K_m)_{EM}}{\rightleftarrows} \mathrm{EMS} \overset{(k_c)_{EM}}{\rightarrow} \mathrm{EM} + \text{products} & \\
K_{EM} \uparrow\downarrow & & \\
 & \mathrm{M} & \\
 & + & \\
 & \mathrm{E} + \mathrm{S} \overset{(K_m)_{E}}{\rightleftarrows} \mathrm{ES} \overset{(k_c)_{E}}{\rightarrow} \mathrm{E} + \text{products} & \\
 & + & \\
 & \mathrm{M} & \\
K_{M} \uparrow\downarrow & & \\
 & \mathrm{ME} + \mathrm{S} \overset{(K_m)_{ME}}{\rightleftarrows} \mathrm{MES} \overset{(k_c)_{ME}}{\rightarrow} \mathrm{ME} + \text{products} & \\
 & + & \\
 & \mathrm{M} & \\
K_{MEM} \uparrow\downarrow & & \\
 & \mathrm{MEM} + \mathrm{S} \overset{(K_m)_{MEM}}{\rightleftarrows} \mathrm{MEMS} \overset{(k_c)_{MEM}}{\rightarrow} \mathrm{MEM} & \\
 & \quad + \text{products} &
\end{array}
$$

$(K_m)_E$ and $(k_c)_M$ continue to be the Michaelis constant and catalytic rate constant governing

product formation as the result of enzyme–substrate complex between thrombin (E) and prethrombin 1 (S). However, interaction of the prothrombin F2-$A_{(1-13)}$ (modifier, M) with the two exosites gives rise to the equilibrium coexistence of three enzyme–modifier complexes, EM, ME and MEM, each of which can react with substrate and hence catalyze the cleavage of prethrombin 1 by its own catalytic rate constant. After allowance is made for the fact that the combined concentration of all enzyme–substrate complexes contributes negligibly to the total enzyme concentration (because $[S]_t \ll K_m$), it follows that the observed first-order rate constants in the presence of modifier, $(k_{obs})_M$, are given by

$$(k_{obs})_M = \Big[(k_c/K_m)_E + [M]\{(k_c/K_m)_{EM}/K_{EM} + (k_c/K_m)_{ME}/K_{ME}\} + [M]^2\{(k_c/K_m)_{MEM}/(K_{ME}K_{MEM})\}\Big][E]_t \Big/ \big(1 + [M](1/K_{EM} + 1/K_{ME}) + [M]^2/(K_{ME}K_{MEM})\big) \quad (3a)$$

$$= \frac{\{(k_c/K_m)_E + A[M] + (B/C)[M]^2\}[E]_t}{1 + D[M] + C[M]^2} \quad (3b)$$

Consequently, although the apparent first-order rate constant in the presence of modifier requires description in terms of the measured rate constant in the absence of modifier, $(k_c/K_m)_E$, and six other parameters, K_{EM}, K_{ME}, K_{MEM}, $(k_c/K_m)_{EM}$, $(k_c/K_m)_{ME}$, and $(k_c/K_m)_{MEM}$, only four independent parameters (polynomial coefficients A, B, C and D) emerge from non-linear regression analysis in terms of Eqs. (3a),(3b). No unequivocal appraisal of the results in terms of even the simplest mechanistic scheme is therefore possible. However, qualitative reasoning based on the inset in Fig. 4 shows that the effector interaction responsible for activation (say, K_{EM}) must be governed by a dissociation constant that may be as large as 10 μM, whereas that responsible for non-competitive inhibition (K_{ME}) clearly is less than 100 μM; and therefore that the thrombin exosites responsible for the two phenomena exhibit affinities for F2-$A_{(1-13)}$ which appear to be of the same order of magnitude. We note that the value of k_{obs} measured in the absence of modifier may also reflect partial activation because of the F2 region in the substrate, prethrombin 1. Extrapolation of values of k_{obs} for a range of prethrombin 1 concentrations to zero concentration would be required to pinpoint the precise extents of activation and ultimate inhibition. Under the conditions of these studies, the consequences (effect on k_c/K_m) of the inhibitory interaction outweigh those of the activation phenomenon, because the fully saturated thrombin–modifier complex (MEM) exhibits only 40% of the catalytic capability of unmodified enzyme (Fig. 4).

Incorporation of the equilibrium coexistence of two isomeric forms of thrombin [47–49] leads to even greater complexity of the model, which then needs to be described in terms of two forms of E, EM, ME and MEM. The quantitative expression for the observed rate constant then assumes the form

$$(k_{obs})_M = \frac{\{(k_c/K_m)_E + Y(k_c/K_m)_{E^*} + H[M] + J[M]^2\}[E]_t}{1 + Y + L[M] + N[M]^2} \quad (4)$$

where $(k_c/K_m)_E$ and $(k_c/K_m)_{E^*}$ are the first-order rate constants for isomers E and E* respectively; and where Y is the isomerization constant describing the equilibrium between anticoagulant (E) and procoagulant (E*) states. The remaining four coefficients (H, J, L and N) contain Y and terms in 12 additional parameters: K_{EM}, K_{ME}, K_{MEM}, $(k_c/K_m)_{EM}$, $(k_c/K_m)_{ME}$, $(k_c/K_m)_{MEM}$, and the corresponding E* series. Because unequivocal appraisal in terms of this more realistic model is clearly out of the question, we again resort to qualitative reasoning. The present detection of two effector binding sites with affinity for F2-$A_{(1-13)}$ (exosites I and II?), and presumably other macromolecular effectors, has increased the complexity of the quantitative analysis of kinetics in terms of the isomerizing thrombin model [47–50]. However, it has elimi-

nated one restriction. Previously it might have been assumed that the effector interaction responsible for curtailing the procoagulant activity also enhanced the anticoagulant activity. There is now no necessity to consider the two controls in terms of the one interaction between effector and thrombin. The possibility that the allosteric switch might be 'thrown' by a macromolecular effector that is generated during thrombin formation provides a biologically plausible mechanism for switching. This possibility deserves further investigation, particularly under conditions in which the procoagulant and anticoagulant forms of thrombin can be made to predominate.

In view of the X-ray crystallographic evidence for F2-$A_{(1-13)}$ interaction with the putative heparin-binding exosite (exosite II) of thrombin [4], and the demonstration that F2-$A_{(1-13)}$ must bind to a site different from the hirugen binding site (exosite I) [44], exosite II is proposed as the most likely structural source of the interaction (Fig. 4) that makes F2-$A_{(1-13)}$ an activator of prethrombin 1 cleavage by thrombin. The source of the noncompetitive inhibition exhibited by F2-$A_{(1-13)}$ (and by the F1 kringle of prothrombin) is suggested to be the consequence of interaction with the fibrinogen-binding exosite of thrombin on the basis of two lines of evidence. First, it was noted in the crystallographic study [4] also, that an interaction between F2-$A_{(1-13)}$ and thrombin could be identified with Arg_{77A} as well as the residues associated with the putative heparin-binding site. Secondly, there is competition between the interactions of F2-$A_{(1-13)}$ and hirudin peptides with an active-site blocked fluorescent thrombin derivative [42]. This adds supporting structural evidence for F2-$A_{(1-13)}$ binding to a region adjacent to the fibrinogen-binding site.

Thrombin is important physiologically in the proteolysis of several critical plasma proteins such as fibrinogen, Factor V, Factor VIII, Factor XIII, Protein C, prothrombin, and Factor XI. Regulation of thrombin, i.e. modulation of thrombin's ability to achieve the required temporal and spatial specificity, clearly involves the formation of complexes with other macromolecular effectors. The fibrinogen-binding site has been implicated in the interaction of thrombin not only with fibrinogen but also with thrombomodulin [7–11,51], the platelet thrombin receptor peptide [6,48,49], Factor V and Factor VIII [52], and F2-$A_{(1-13)}$ (Fig. 4 of present study). It is interesting to conjecture that the binding of F2-$A_{(1-13)}$ (or any species containing the F2-$A_{(1-13)}$ fragment) to the putative heparin-binding site of thrombin might modulate the competition between various other macromolecular effectors for the fibrinogen-binding site. When the other receptor binding actions of thrombin [6], and the effects of the platelet thrombin receptor [46] and thrombomodulin growth factor domains on thrombin specificity [51] are taken into consideration, it seems likely that macromolecular effector modulation of thrombin specificity may be more important than the extent of complementarity between substrate and the enzyme active site in determining the substrates on which thrombin preferentially acts.

The most important contribution of the present investigation is probably not so much the demonstration of thrombin activation and inhibition by fragment 2 specifically, but rather the consequent inference that the specificity of thrombin toward its many substrates may be altered through multiple exosite interactions by the same effector. For example, selective modulation of thrombin that would initially increase, then decrease thrombin efficiency in activating Factor V, and coincident with the latter process enhance its efficiency in the activation of Protein C, is but one example of an avenue worthy of further investigation.

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