Proteolytic Formation of Either of the Two Prothrombin Activation Intermediates Results in Formation of a Hirugen-binding Site*

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Hirugen, a synthetic dodecapeptide corresponding to the carboxyl-terminal amino acids 53–64 of hirudin, binds within a deep groove in thrombin that contains a cationic region referred to as the anion-binding exosite. This region is important in many of the binary interactions of thrombin with macromolecular substrates and cofactors. Fluorescein-labeled hirugen was used to probe which steps in the prothrombin activation process generate this anion-binding exosite. Two activation cleavage sites exist in bovine prothrombin. Cleavage at Arg^{231}-Thr^{275} releases the activation fragments to generate the thrombin precursor, prethrombin 2. Cleavage of prothrombin within a disulfide loop at Arg^{283}-Ile^{324} leads to formation of meizothrombin with no loss of peptide material but with formation of amidolytic activity. Cleavage of the same bond in prethrombin 2 generates thrombin. Hirugen, labeled at the amino terminus with fluorescein isothiocyanate, does not bind to prothrombin but does bind to thrombin ($K_d = 9.6 \pm 1.2 \times 10^{-7}$ M), prethrombin 2 ($K_d = 1.3 \pm 0.1 \times 10^{-7}$ M), thrombin-fragment 2 complex ($K_d = 1.1 \pm 0.2 \times 10^{-7}$ M), and meizothrombin ($K_d = 1.6 \pm 0.5 \times 10^{-7}$ M). Prothrombin fragment-2 and hirugen both bind independently to thrombin. A ternary complex can form with hirugen and fragment-2 and either thrombin or prethrombin 2, suggesting that fragment-2 and hirugen bind to discrete sites. Hirugen also alters the active site conformation of thrombin as detected by modulation of synthetic substrate hydrolytic activity. These studies suggest that conformational changes, rather than alleviating steric hindrance, are responsible for the formation of the hirugen-binding site during prothrombin activation. Furthermore, this conformational change can be effected by the cleavage of either of the two bonds required for activation of prothrombin.

During prothrombin activation, at least two bonds must be cleaved, giving rise to two possible intermediates. Both intermediates can be isolated, and they have distinct functional characteristics (1, 2). Prethrombin 2, a single chain precursor of thrombin with no proteolytic or amidolytic activity, results from proteolytic cleavage at Arg^{231}-Ile^{244} to release the activation fragments that constitute nearly one-half of the prothrombin molecule. Prethrombin 2 is converted to thrombin by cleavage of the Arg^{231}-Ile^{244} bond within a disulfide loop (3). Both thrombin and prethrombin 2 interact noncovalently with activation fragment-2. This interaction augments prethrombin 2 activation (4) and enhances thrombin's esterolytic activity (5). Meizothrombin is formed from prothrombin by the cleavage of the Arg^{231}-Ile^{244} bond, but because the cleavage occurs within a disulfide loop, no activation fragments are released. Although meizothrombin has activity toward low molecular weight substrates, it has little activity toward fibrinogen (6). One explanation for the lack of clotting activity is that the extended fibrinogen-binding pocket is not yet available in meizothrombin, perhaps because the activation fragment masks the extended fibrinogen-binding pocket, referred to as the anion-binding exosite. No direct studies have been performed to evaluate this hypothesis.

A useful reagent to probe this issue was suggested by recent studies on the function of different domains of the leech thrombin inhibitor hirudin (7, 8). Of particular importance to the present study, the carboxyl-terminal 12-residue portion of hirudin, referred to as hirugen, binds to thrombin and inhibits fibrinogen clotting activity without inhibiting hydrolytic activity toward low molecular weight substrates (9, 10). Thus, the hirugen-thrombin complex shares many properties in common with meizothrombin. X-ray crystallographic data of the thrombin-hirudin complex indicate that the hirugen portion of the hirudin molecule binds to the deep groove that appears to include the anion-binding exosite (11). We have used hirugen to determine which proteolytic events in prothrombin activation are associated with formation of the anion-binding exosite.

**EXPERIMENTAL PROCEDURES**

Chemicals and Proteins—Chromogenic substrates S-2222, S-2238, and S-2288 were from Helena Laboratories, Beaumont, TX; the Spectrozyme substrates SPC1-E, SPXa, SPPCa, and SPTH were kindly provided by American Diagnostics Inc., Greenwich, CT; Chromozym Xa, Chromozym tPA, and Chromozym I were from Boehringer Mannheim; and N-benzoyl-Val-Gly-Arg-p-nitroanilide and N-p-tosyl-Gly-Pro-Arg-p-nitroanilide were from Sigma. Fluorescein 5-isothiocyanate (FITC) was purchased from Molecular Probes, Inc., Eugene, OR. D-Phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PACAK) was from Calbiochem (La Jolla, CA). D-Phenylalanine-1-prolyl-1-arginine chloromethyl ketone (PPACK) was a gift from Dr. W.W. Huizinga, Erasmus University, Rotterdam, The Netherlands. Bivalirudin was a gift from Dr. M. Steinberg, Lilly Research Laboratories, Indianapolis, IN. Dipeptidyl peptidase IV (DPP IV) was a gift from Dr. Frans J.J. van Oort, University of Maastricht, Maastricht, The Netherlands. Shear was provided by Mr. D. Stevens, Dr. A. Abshire, and Dr. L. Lamey of the Texas A&M Agricultural Research and Extension Center, College Station, TX.
of FI-hirugen decreased upon binding to thrombin, prethrombin 2, hirugen. Thrombin had the same low mobility as prethrombin 2. For these proteins. Titrations were performed by the sequential addition of 8 nm. Reaction 6, 25 pg of thrombin was isolated by published methods (1). HIrugen was further purified on a Vydac C18 218TP54 reverse phase column (4.6 x 300 mm, 5 µM/30 A) attached to an fast protein liquid chromatography system (Pharmacia LKB Biotechnology Inc.). In experiments with prethrombin 2, the column was equilibrated and eluted with 10 mM sodium phosphate, pH 7.0, from 0 to 80% acetonitrile. FI-hirugen was stored at -80 °C. The concentration of FI-hirugen was estimated assuming a molar absorptivity of 6.8 x 10^4 M^-1 cm^-1 at 492 nm for covalent FITC conjugates (13)."
Hirugen Interaction with Prothrombin Activation Intermediates

![Complex formation of fragment-2 and Fl-hirugen with thrombin and prothrombin activation intermediates.](image)

Fig. 1. Complex formation of fragment-2 and Fl-hirugen with thrombin and prothrombin activation intermediates. The TSK-250 column was equilibrated in 0.15 M NaCl, 10 mM phosphate, pH 7.5 (A–H) and in 0.1 M NaCl, 20 mM HEPES, pH 7.5 (I–J). A transfers (---), relative fluorescence intensity at 515 nm ( - - - ). The scale at the bottom indicates the retention volume in milliliters. The following samples (100 μl) were applied to the column. A, thrombin (20 μM); B, thrombin (20 μM) + Fl-hirugen (10 μM); C, Fl-hirugen (10 μM); D, fragment-2 (20 μM); E, thrombin (20 μM) + fragment-2 (20 μM) + Fl-hirugen (10 μM); F, thrombin (20 μM) + fragment-2 (40 μM) + Fl-hirugen (10 μM); G, meizothrombin (20 μM) + Fl-hirugen (10 μM); H, meizothrombin des fragment-1 (20 μM) + Fl-hirugen (10 μM); I, prethrombin 2 (20 μM); J, prethrombin 2 (20 μM) + fragment-2 (40 μM) + Fl-hirugen (10 μM).

The Arg121/Ile124 bond and subsequent release of the activation fragment was also investigated. Fl-hirugen bound to PPACK meizothrombin or PPACK meizothrombin des fragment-1 (Fig. 1, G and H). Similar gel filtration experiments with prothrombin and prethrombin 1 failed to detect any Fl-hirugen binding (data not shown). Thus, neither the formation of catalytic activity nor the release of the covalently associated activation fragments are required for hirugen binding, but the binding site is cryptic in prothrombin.

Additional evidence that hirugen does not prevent fragment-2 thrombin and fragment 2-prethrombin 2 complex formation was obtained by electrophoresis studies. When subjected to polyacrylamide gel electrophoresis in the absence of detergents under the conditions described under “Experimental Procedures,” thrombin and prethrombin 2 migrate slowly, fragment-2 migrates rapidly, and the complex is intermediate. A 70-fold molar excess of hirugen did not disrupt fragment-2 complex formation with either thrombin or prethrombin 2, and hirugen itself did not alter the electrophoretic mobility of either thrombin or prethrombin 2 in the absence of fragment-2 (data not shown). Attempts to provide direct evidence of a ternary complex of fragment-2-thrombin-hirugen using Fl-hirugen were negative, probably because the off rate was too high to even allow visualization of the thrombin-Fl-hirugen complex alone.

Fluorescence Analysis of Fl-hirugen Binding—To ascertain the affinities of Fl-hirugen binding to thrombin, prethrombin 2, and PPACK-meizothrombin derivatives, we utilized the observation that Fl-hirugen binding is associated with a decrease in fluorescein emission intensity. The titration curves are shown in Fig. 2, and the maximum emission intensity changes and calculated Kd values for each of the protein-fl-hirugen interactions are summarized in Table I. It is apparent that all forms of the thrombin intermediates and thrombin itself bound Fl-hirugen saturably and with moderately high affinity. In all cases, inclusion of a 60–100-fold molar excess of unlabeled hirugen over the thrombin or thrombin intermediate reversed greater than 94% of the emission intensity change, thereby indicating that Fl-hirugen binding was specific (data not shown). The observation that hirugen binding was 10-fold weaker in the presence of fragment-2 suggested the possibility that there could be some displacement of fragment-2 during the titrations. If this were the case, then the Fl-hirugen binding affinity would be different at different saturating concentrations of fragment-2. The binding was monitored at 2- and 5-fold molar excesses of fragment 2. The titration curves were indistinguishable, indicating that displacement of fragment-2 is an unlikely explanation for the 10-fold reduction in binding affinity (data not shown). The observation that the maximum change in Fl-hirugen fluorescence is greater with the fragment-2-thrombin complex than with thrombin alone also indicates that Fl-hirugen can bind to form a ternary complex.

![Fluorescence-detected binding of Fl-hirugen to thrombin and thrombin precursors at equilibrium.](image)

Fig. 2. Fluorescence-detected binding of Fl-hirugen to thrombin and thrombin precursors at equilibrium. The initial Fl-hirugen concentration was 7 nM. Panel A, thrombin; Panel B, prethrombin 2; Panel C, thrombin titrated into 10 μM fragment-2; Panel D, meizothrombin.

| Protein species | 1 – Fl/F0 | Kd nM | n |
|----------------|-----------|------|---|
| Thrombin       | 18.6 ± 3.2| 9.6 ± 1.2 × 10^-6 | 6 |
| Prethrombin 2  | 18.2 ± 1.4| 1.3 ± 0.1 × 10^-7 | 4 |
| Meizothrombin  | 8.3 ± 1.6 | 1.6 ± 0.5 × 10^-6 | 4 |
| Meizothrombin des fragment-1 | 14.4 ± 3.3 | 4.7 ± 1.1 × 10^-6 | 4 |
| Thrombin + fragment-2 | 26.2 ± 3.0 | 1.1 ± 0.2 × 10^-6 | 8 |
The Influence of Hirugen on Chromogenic Substrate Hydrolysis—The amidolytic activity of human thrombin was modified upon interacting with hirugen. For example, the rate of cleavage of Spectrozyme TH at saturating substrate (4 × 10^{-7} M) was increased 57% by hirugen (Fig. 3). In contrast, hirugen inhibited the hydrolysis of several substrates (Fig. 3). To facilitate the analysis of hirugen effects on prothrombin activation, it was particularly useful to identify substrates that were unaffected by hirugen. Under our experimental conditions, hirugen had almost no effect on the hydrolysis rates of tosyl-Gly-Pro-Arg-p-nitroanilide and benzoyl-Ile-Glu-Gly-Arg-p-nitroanilide. The results with bovine thrombin were similar, except that none of the substrates proved insensitive to the presence of hirugen (data not shown).

The Influence of Hirugen on the Activation of Prothrombin 2—The rate of activation of human prethrombin 2 by factor Xa and factor Va remained essentially constant up to about 1 μM hirugen (Fig. 4). At higher hirugen concentrations, there was a dose-dependent decrease in the initial rate of prothrombin 2 activation. In the presence of fragment-2, inhibition by hirugen required higher concentrations (Fig. 4, line 2). Hirugen also inhibited prothrombin activation under these conditions with an IC_{50} ≈ 10 μM (data not shown). By using a synthetic substrate (that is least affected by hirugen (N-p-tosyl-Gly-Pro-Arg-p-nitroanilide), the observed effect of hirugen on thrombin was minimized (Fig. 4, line 1). In the absence of factor Va in the activation mixture, hirugen failed to inhibit prethrombin 2 activation at concentrations as high as 1 mM (Fig. 4, line 2).

DISCUSSION

In prothrombin and prethrombin 1, the hirugen-binding site is not expressed, but the binding site is expressed in either of the two possible activation intermediates, as well as thrombin itself. This conclusion is based primarily on direct binding studies employing gel filtration chromatography and by equilibrium methods employing FI-hirugen. Fragment-2 can interact with either thrombin or prethrombin 2 without displacing FI-hirugen. The equilibrium methods are not unambiguous, however, since we have no independent means of monitoring fragment 2 binding in these experiments. The observation that a F_{max} for FI-hirugen binding to thrombin is greater in the presence than in the absence of fragment 2 further supports the conclusion that a ternary complex can form. The nearly equal affinity of meizothrombin and thrombin for FI-hirugen indicate that the fragment-2- and hirugen-binding sites are essentially nonoverlapping. Since fragment-2 does not block hirugen binding, fragment-2 and hirugen cannot bind in the same location either in meizothrombin or the thrombin-fragment 2 complex. These results suggest that formation of the hirugen-binding site that accompanies either of the two activation cleavage events in prothrombin results from conformational changes rather than unmasking the hirugen-binding site by alleviating steric hindrance due to fragment-2 interaction. The latter conclusions are predicated on the assumption that reversible association of fragment-2 with thrombin or prethrombin 2 and the interaction of the fragment-2 and thrombin domains within meizothrombin involve the same site as in prothrombin. In drawing these conclusions, it is important to note that the affinity of meizothrombin for FI-hirugen was slightly higher than that of thrombin, whereas the affinity of prethrombin 2 for FI-hirugen was slightly lower. These differences probably reflect differences in the conformation of the hirugen-binding site in these species. Noncovalent association of the activation peptide with thrombin and prethrombin 2 (data not shown) decrease the affinity of FI-hirugen binding, indicating that the conformation of the hirugen-binding cleft is different when fragment 2 associates noncovalently or covalently with thrombin and prethrombin 2, although limited overlap of the binding sites cannot be totally excluded.
Fluorescein labeling of hirugen allowed quantitative assessment of binding affinity, but created the potential problem of altering the binding specificity (14, 15). The ability of unlabeled hirugen to displace Fl-hirugen strongly argues against this possibility. The affinity for Fl-hirugen observed in this study ($K_a = 9.8 \times 10^{-8}$ M using bovine thrombin) was somewhat higher than the previously reported values for hirugen. $K_a = 1.44 \times 10^{-7}$ M in blocking human thrombin fibrinogen clotting (16); $K_a = 5.4 \times 10^{-7}$ M in human thrombin-catalyzed release of fibrinopeptide A from fibrinogen (9). This difference is somewhat greater than it would appear since the affinity of human thrombin for hirugen is approximately 10-fold greater than that of bovine thrombin (17, 18).

Not only does the hirugen-binding site result from conformational changes that occur during prothrombin activation, but interaction of hirugen with thrombin also elicits a conformational change that alters substrate specificity (9, 19). Our experiments, there was a very small (10%) increase in the $K_M$, inhibition does not occur at hirugen concentrations up to 1 mM. The hirugen concentration dependence for inhibition of prothrombin 2 activation was somewhat similar to that observed for binding to prethrombin 2. Thus, the observed inhibition could reflect either that hirugen inhibits factor Va-Xa interaction or that the hirugen-prethrombin 2 complex is a poor substrate for the factor Xa-Va complex. In many experiments, there was a very small (10%) increase in the prethrombin 2 activation rate at hirugen concentrations somewhat lower than the prethrombin 2 concentration. This increase occurred only in the presence of factor Va and the absence of fragment 2 (data not shown). The latter experiments favor the concept that the hirugen may dissociate the factor Xa-Va complex, although definitive evidence will require direct equilibrium measurements.

The demonstration in the present study that the hirugen-binding site is present in prethrombin 2 complements the finding (3) that the active site titrant, dansyl-arginine-N-(3-ethyl-1,5-pentanediyl)amide, can interact with prethrombin 2. The presence of the hirugen site in prethrombin 2 helps to explain the observation that prethrombin 2 binds to thrombomodulin, albeit approximately 10-fold weaker than thrombin (20). Thus, several of the ligand-binding sites of thrombin are already expressed on prethrombin 2, although its catalytic triad is not yet competent.

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