Inhibition of Meizothrombin and Meizothrombin(desF1) by Heparin Cofactor II*

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Meizothrombin and meizothrombin(desF1) are intermediates formed during the conversion of prothrombin to thrombin by factor Xa, factor Va, phospholipids, and Ca\(^{2+}\) (prothrombinase). These intermediates are active toward synthetic peptide substrates but have limited ability to interact with platelets or macromolecular substrates such as fibrinogen. Meizothrombin and meizothrombin(desF1) activate protein C, however, and may exert primarily an anticoagulant effect. In this study, we investigated the inhibition of meizothrombin and meizothrombin(desF1) by two glycosaminoglycan-dependent protease inhibitors, heparin cofactor II (HCII) and antithrombin (AT). Purified recombinant meizothrombin and meizothrombin(desF1) were inhibited by HCII in the presence of dermatan sulfate with maximal second-order rate constants of 8 \times 10^6 M\(^{-1}\)min\(^{-1}\) and 1.8 \times 10^6 M\(^{-1}\)min\(^{-1}\), respectively, but were inhibited less than one-tenth as fast by AT in the presence of heparin. Similarly, the products of the prothrombinase reaction were inhibited in situ more effectively by HCII than by AT. When HCII and dermatan sulfate were present continuously during the prothrombinase reaction, meizothrombin was trapped as a sodium dodecyl sulfate-stable complex with HCII and no amidolytic activity could be detected with a thrombin substrate. Our findings indicate that HCII is an effective inhibitor of meizothrombin and meizothrombin(desF1) and, therefore, might regulate the anticoagulant activity of these proteases.

Thrombin is a key enzyme in several biological processes, including blood coagulation, wound healing, and inflammation (1). Factor Xa converts human prothrombin to thrombin by cleavage of the peptide bonds following Arg-271 and Arg-320 (2). The order in which these bonds are cleaved depends on the assembly of the prothrombinase complex. In the presence of factor Xa and Ca\(^{2+}\), prothrombin is first cleaved after Arg-271, giving rise to fragment 1-2 and prethrombin 2 (3). When factor Xa and its cofactor, factor Va, are assembled on a membrane surface in the presence of Ca\(^{2+}\), factor Xa first cleaves after Arg-320, giving rise to meizothrombin (4, 5). Cleavage of the second factor Xa-sensitive bond in either prethrombin 2 or meizothrombin yields thrombin. Meizothrombin has been shown recently to be a major intermediate formed during coagulation of whole blood in vitro (6).

Meizothrombin retains the N-terminal \(\gamma\)-carboxyglutamic acid domain of prothrombin, which enables it to bind to membrane phospholipids in the presence of Ca\(^{2+}\). Cleavage of the thrombin-sensitive peptide bond following Arg-155 in meizothrombin removes the \(\gamma\)-carboxyglutamic acid domain and generates meizothrombin(desF1) (7). In contrast to prothrombin or prethrombin 2, meizothrombin and meizothrombin(desF1) hydrolyze synthetic peptide substrates at rates comparable with that of thrombin (8–10). Meizothrombin and meizothrombin(desF1) are much less active with respect to fibrinogen clotting, platelet activation, and activation of the thrombin-activable fibrinolysis inhibitor (9–11); therefore, these intermediates have greatly reduced procoagulant and anti-fibrinolytic activity in comparison with thrombin. However, meizothrombin and meizothrombin(desF1) are activated by protein C, which produces a protein similar to thrombin (19, 20). X-ray crystallographic studies of thrombin complexed with fragment 2 suggest that exosite II is occupied by the fragment 2 domain in meizothrombin or meizothrombin(desF1) (21). This structural model is consistent with the observation that meizothrombin is not inhibited rapidly by AT in the presence of heparin (11). Because exosite II is not involved in the inhibition of thrombin by HCII in the presence of dermatan sulfate (22), HCII could potentially inhibit meizothrombin and meizothrombin(desF1) better than AT. We now present evidence to support this hypothesis.

The abbreviations used are: AT, antithrombin; HCII, heparin cofactor II; PPACK, Phe-Pro-Arg-chloromethylketone; STI, soybean trypsin inhibitor; PAGE, polyacrylamide gel electrophoresis; PS, phosphatidylserine; PC, phosphatidylcholine.
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EXPERIMENTAL PROCEDURES

Materials—Recombinant human meizothrombin and meizothrombin(desF1) were expressed in baby hamster kidney cells and purified as described previously (23). Human α-thrombin, prothrombin, factor Va, factor Xa, and biotinylated Phe-Pro-Arg-chloromethylketone (PKC) were purchased from Haematologic Technologies (Essex Junction, VT). The prothrombin activator from Echis carinatus venom (Ecarin) was purchased from American Diagnostics (Greenwich, CT). HCII and AT were purified from human plasma and characterized as described previously (15). Bovine lung heparin was obtained from Upjohn (Kalamazoo, MI), porcine skin dermatan sulfate, and soybean trypsin inhibitor (STI) from Sigma (St. Louis, MO), phosphatidylserine (PS), and phosphatidylycholine (PC) from Avanti Polar Lipids (Alabaster, AL), and tosyl-Gly-Pro-Arg-nitroanilide from Boehringer Mannheim. Human factor Xa (R271A) was used to generate a stable form of meizothrombin (23). Similarly, a stable form of meizothrombin(desF1) was obtained from recombinant prothrombin (R271A, R284A). The enzymatic activities and calcium- and phospholipid-binding properties of recombinant meizothrombin and meizothrombin(desF1) are similar to those of the native enzymes (10, 23).

Figs. 1 and 2. Inhibition of recombinant meizothrombin and meizothrombin(desF1) by HCII and AT. HCII (90 nM) or AT (140 nM) was incubated with meizothrombin or meizothrombin(desF1) (10 nM) in the absence (●) or presence (○) of a glycosaminoglycan (50 μg/ml). The glycosaminoglycans used were dermatan sulfate with HCII and heparin with AT. At various times, the remaining thrombin activity was determined by hydrolysis of tosyl-Gly-Pro-Arg-nitroanilide (ΔA405/min). Each line represents the best fit of the data to the exponential equation given under “Experimental Procedures.”

Inhibition of Recombinant Proteases—Inhibition of recombinant meizothrombin or meizothrombin(desF1) was studied in the presence of 9-fold molar excess of HCII or AT. The inhibitor, glycosaminoglycan, and enzyme were incubated at room temperature in 100 μl of TS/P buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM polyethylene glycol, pH 7.4) containing 5 mM CaCl2. Some incubations also included 50 μM PC:PS vesicles (3:1) prepared as described elsewhere (24). Reactions were stopped at various times by the addition of 500 μl of the chromogenic substrate tosyl-Gly-Pro-Arg-nitroanilide (100 μM in TS/P buffer). The absorbance at 405 nm was recorded every 5 s for 100 s to determine the residual enzyme activity (E). The pseudo-first-order rate constant (k') was determined by fitting the experimental data to the equation E0 = E + k't, where E0 is the initial enzyme activity and k't is the activity at time t. The second-order rate constant (k) was calculated from the equation k = k'/[I], where [I] is the initial inhibitor concentration.

Formation of Protease-HCII Complexes during the Prothrombinase Reaction—Prothrombin (1.4 μM) was activated by prothrombinase (0.3 nM factor Xa, 5 nM factor Va, 10 μM PC:PS vesicles, and 5 mM CaCl2) in the presence of HCII (1.5 μM) and dermatan sulfate (50 μg/ml). Samples were withdrawn at various times, subjected to 7.5% SDS-PAGE under non-reducing conditions, and stained with Coomassie Blue.

Conversion of Meizothrombin-HCII to Thrombin-HCII by Prothrombinase—Meizothrombin-HCII complexes were generated by incubating prothrombinase (1 μM) with Ecarin (1 Ecarin unit/ml) (7) in the presence of HCII (2 μM) and dermatan sulfate (100 μg/ml) in TS/P buffer containing 5 mM CaCl2. After a 1-h incubation, an equal volume of prothrombinase (0.3 nM factor Xa, 12 nM factor Va, 100 μM PC:PS vesicles, and 5 mM CaCl2) in TS/P buffer, or buffer containing 5 mM CaCl2 only, was added. Samples were withdrawn at various times, subjected to 6.5% SDS-PAGE under non-reducing or reducing conditions, and stained with Coomassie Blue. Bands were quantified by densitometry as described above.

RESULTS

Native meizothrombin is stable only in the presence of a thrombin active site inhibitor. In the absence of an inhibitor, thrombin-sensitive peptide bonds after Arg-155 or Arg-284 can be cleaved to yield meizothrombin or thrombin,2 respectively (Fig. 1). In the present study, recombinant prothrombin with modified cleavage sites for thrombin (R155A, R284A) and factor Xa (R271A) was used to generate a stable form of meizothrombin (23).

2 This product is identical to the stable form of human thrombin (α-thrombin) that normally results from autolytic cleavage after Arg-284 in the A-chain.
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HCII most rapidly in the presence of 6–40 μg/ml heparin, but the maximal second-order rate constants were less than one-tenth of those observed with HCII plus dermatan sulfate.

Table I compares the second-order rate constants for inhibition of meizothrombin and meizothrombin(desF1) in the absence or presence of a glycosaminoglycan (50 μg/ml) with the rate constants observed for thrombin at optimal glycosaminoglycan concentrations. HCII inhibits thrombin at about one-tenth the rate of AT in the absence of a glycosaminoglycan (17). In agreement with previous work (11), AT inhibited thrombin more rapidly than meizothrombin or meizothrombin(desF1) in the absence of heparin. Similarly, the basal rate of inhibition of thrombin by HCII was slightly more rapid than that of meizothrombin or meizothrombin(desF1). Dermatan sulfate increased the rate at which HCII inhibited meizothrombin by 380-fold, meizothrombin(desF1) by 1000-fold, and thrombin by −16,000-fold. Heparin had a similar effect. By contrast, heparin accelerated inhibition of meizothrombin and meizothrombin(desF1) by AT only 4- to 11-fold. During prothrombin activation, meizothrombin remains bound to the phospholipid membrane by means of its γ-carboxyglutamic acid domain. However, addition of PC:PS vesicles and Ca++ did not diminish the ability of HCII to inhibit recombinant meizothrombin in the presence of dermatan sulfate or heparin (Table I).

To compare the ability of HCII and AT to inhibit the products of the prothrombinase complex in situ, prothrombin was activated by factor Xa in the presence of factor Va, PC:PS vesicles, and Ca++

Under the conditions of this experiment, amidolytic activity determined with a thrombin substrate increased linearly up to 5 min, at which time <10% of the prothrombin was activated (Fig. 4A). The reaction products were monitored with biotinylated PPACK, which quantitatively labels the active sites of meizothrombin, meizothrombin(desF1), and thrombin (Fig. 4B) (6). After 5 min, the predominant products were thrombin (74%) and meizothrombin(desF1) (23%), but a small amount of meizothrombin (3%) was also detectable. In parallel experiments, STI was added at 5 min to inhibit the factor Xa.

Heparin and dermatan sulfate (or AT and heparin) were then added, and the amidolytic activity was followed over time (Fig. 4A). In agreement with a previous study (26), inhibition of the amidolytic activity by AT plus heparin occurred in a biphasic manner, such that ~56% of the activity was inhibited at the earliest time point and the remainder decayed with a second-order rate constant of 2.0 × 10^6 m⁻¹·min⁻¹. In marked contrast, inhibition of the amidolytic activity by HCII either in the presence of dermatan sulfate or heparin was nearly complete at the earliest time point, which indicates that the reaction occurred with a rate constant >2.7 × 10^7 m⁻¹·min⁻¹. In the absence of a glycosaminoglycan, the amidolytic activity was inhibited in a monophasic manner by AT, with a rate constant of 5.1 × 10^5 m⁻¹·min⁻¹, whereas HCII inhibited the activity much more slowly.

In the experiment shown in Fig. 5, HCII and dermatan sulfate were present continuously during prothrombin activation by factor Xa, factor Va, PC:PS vesicles, and Ca++. In contrast to the preceding experiment (Fig. 4), no amidolytic activity was detected with tosyl-Gly-Pro-Arg-p-nitroanilide during the 20-min incubation (data not shown). The reaction mixture was sampled at various times and analyzed by SDS-PAGE under non-reducing conditions. The gel shows that meizothrombin and thrombin were trapped as SDS-stable complexes with HCII. Notably, HCII-meizothrombin(desF1) complexes did not accumulate, which indicates that HCII prevented the proteolytic conversion of meizothrombin to meizothrombin(desF1).

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**Table I**

**Second-order rate constants for inhibition of thrombin intermediates by HCII and AT**

Incubations were performed under pseudo first-order conditions, and rate constants were determined as described under “Experimental Procedures.” In experiments with meizothrombin or meizothrombin(desF1), the glycosaminoglycan was present at a final concentration of 50 μg/ml. In experiments with thrombin, the concentration of heparin was 10 unit/ml (~67 μg/ml) with HCII and 1 unit/ml (~7 μg/ml) with AT, and the concentration of dermatan sulfate was 100 μg/ml.

| Protease          | Inhibitor          | Glycosaminoglycan         | None | Heparin | Dermatan sulfate |
|-------------------|--------------------|---------------------------|------|---------|------------------|
| Meizothrombin     | HCII               |                           | 5.9  | 6.3     |                  |
|                   | HCII (PCPS)        |                           | 5.9  | 6.3     |                  |
|                   | AT                 |                           | 5.9  | 6.3     |                  |
| Meizothrombin(desF1)| HCII             |                           | 5.9  | 6.3     |                  |
|                   | AT                 |                           | 5.9  | 6.3     |                  |
| Thrombin          | HCII               |                           | 5.9  | 6.3     |                  |
|                   | AT                 |                           | 5.9  | 6.3     |                  |

**Note:**

- The -fold increase was calculated by dividing the rate determined in the presence of the glycosaminoglycan by the rate determined in the absence of a glycosaminoglycan for a given protease/inhibitor pair.
- Incubations included 50 μM PC:PS vesicles.
- Data from Sheehan et al. (17).
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FIG. 4. In situ inhibition of prothrombin activation products by HCII or AT. A, prothrombin (0.2 μM) was activated by factor Xa (3 μM) in the presence of factor Va (0.6 nM), PC:PS vesicles (50 μM), and CaCl₂ (5 mM). The factor Xa was inactivated at 5 min with STI (0.33 mg/ml) (dashed line), and HCII (105 nM) or AT (105 nM) with or without a glycosaminoglycan (50 μg/ml) was added immediately thereafter. At various times, samples were assayed for amidolytic activity with tosyl-Gly-Pro-Arg-p-nitroanilide. ○, prothrombin activation prior to addition of STI; □, no serpin or glycosaminoglycan added; ■, AT; □, AT plus heparin; ▲, HCII; △, HCII plus dermatan sulfate; ●, HCII plus heparin. Neither heparin nor dermatan sulfate by itself had an appreciable effect on the amidolytic activity before or after addition of STI. B, prothrombin was activated as in panel A without addition of STI, serpin, or glycosaminoglycan. At various times, samples were mixed with biotinylated PPACK (50 μM) and subjected to SDS-PAGE under non-reducing conditions. The gel was then blotted and probed with avidin linked to horseradish peroxidase as described under “Experimental Procedures.”

FIG. 5. Trapping of meizothrombin by HCII and dermatan sulfate during prothrombin activation. Prothrombin (1.4 μM) was incubated for 0.5–20 min with prothrombinase (0.3 nM factor Xa, 5 nM factor Va, 10 μM PC:PS vesicles, and 5 mM CaCl₂) in the presence of HCII (1.5 μM) and dermatan sulfate (50 μg/ml). In separate incubations, HCII and dermatan sulfate were reacted with purified thrombin, recombinant meizothrombin(desF1), or recombinant meizothrombin. Samples were subjected to SDS-PAGE under non-reducing conditions. The gel was stained with Coomassie Blue. The positions of covalent HCII-thrombin complexes are indicated. Pro, prothrombin; DS, dermatan sulfate; Th, thrombin; Mz, meizothrombin.

There are two possible origins of the thrombin-HCII complexes shown in Fig. 5. Either they resulted from cleavage of meizothrombin-HCII by factor Xa, or some of the meizothrombin was converted to thrombin before being inhibited by HCII.

To determine if meizothrombin-HCII complexes can be cleaved by factor Xa, we used the prothrombin activator from E.carinatus venom, which cleaves only the bond after Arg-320 (see Fig. 1) (7), to generate meizothrombin in the presence of HCII and dermatan sulfate. Under these conditions, >90% of the prothrombin was activated, and the resulting meizothrombin formed complexes with HCII. The complexes were then incubated for 0.5–120 min with factor Xa, factor Va, PC:PS vesicles, and Ca²⁺, and the reaction products were analyzed by SDS-PAGE (Fig. 6). Progressive conversion of meizothrombin-HCII to thrombin-HCII was observed under non-reducing conditions (panel A, −DTT). Under reducing conditions (panel A, +DTT), the thrombin B-chain-HCII complex was observed as expected at all time points (the ~35-kDa F1-F2 and ~40-kDa F1-F2-A-chain polypeptides are not shown). Quantification of the complex bands indicated a tₕ/₂ of ~20 min for meizothrombin-HCII in the presence of prothrombinase (Fig. 6B, circles). The complex was stable for at least 120 min in the absence of prothrombinase (Fig. 6B, squares).

DISCUSSION

The proposed mechanism by which HCII inhibits thrombin differs from that of AT as shown in Fig. 7 (for review, see Ref. 13). HCII contains an N-terminal acidic domain that is similar in amino acid composition to the C-terminal portion of hirudin, which binds to anion-binding exosite I of thrombin (27, 28). When dermatan sulfate or heparin binds to HCII, the N-terminal acidic domain is thought to be displaced from the glycosaminoglycan-binding site and to become free to interact with...
thrombin (16, 29, 30). These initial interactions enable thrombin to attack the reactive site peptide bond of HCII more efficiently, leading to rapid formation of the stable HCII-thrombin complex. Thus, glycosaminoglycans accelerate the ability of HCII to inhibit thrombin by an allosteric mechanism that does not require binding of the glycosaminoglycan to thrombin (22). In contrast, heparin catalyzes the thrombin-AT reaction by a template mechanism that requires binding of heparin to anion-binding exosite II of thrombin (19, 20).

The structure of the noncovalent complex of fragment 2 and thrombin determined by x-ray crystallography suggests that anion-binding exosite II is inaccessible in meizothrombin or meizothrombin(desF1) (21). Indirect evidence also suggests that meizothrombin does not bind tightly to heparin (31). The inaccessibility of exosite II in meizothrombin and meizothrombin(desF1) probably explains the failure of heparin to accelerate inhibition by AT significantly. In contrast, dermaban sulfate accelerates inhibition of these intermediates by HCII 380 to 1000-fold (Table I), and the maximum rate of inhibition of meizothrombin by HCII exceeds that of AT by more than 1 order of magnitude. Moreover, the rate is somewhat higher in the presence of phospholipid vesicles and Cu²⁺, which suggests that meizothrombin bound to physiologic membrane surfaces may be susceptible to inhibition by HCII. Despite the relatively rapid rate of inhibition of meizothrombin by HCII in comparison with AT, HCII inhibits thrombin ~50 times faster than meizothrombin. This difference could be explained by conformational changes that occur in the active site when meizothrombin is converted to thrombin.

Cleavage of meizothrombin at Arg-271 produces F1 and thrombin, which bind to each other with high affinity (Kₐ ~ 1 nM) and remain associated, at least temporarily (32, 33). Like meizothrombin and meizothrombin(desF1), the non-covalent F1-thrombin complex may resist inhibition by AT. To explore this possibility, we determined the kinetics of inhibition of the amidolytic activity generated at an early stage of the prothrombinase reaction, when the ratio of thrombin (or the non-covalent F1-thrombin complex) to meizothrombin(desF1) was ~3:1 (Fig. 4). The results were in reasonable agreement with the kinetics of inhibition of purified thrombin and meizothrombin(desF1) summarized in Table I, if one assumes either that AT plus heparin inhibits F1-thrombin and thrombin at approximately the same rate or that F1-thrombin dissociates during the course of the experiment. This experiment also shows that meizothrombin(desF1) generated from native human prothrombin is inhibited more rapidly by HCII than by AT, in agreement with the results obtained with recombinant meizothrombin(desF1).

When present during the prothrombinase reaction, HCII traps meizothrombin as SDS-stable meizothrombin-HCII complexes (Fig. 5). This observation indicates that factor Xa does not convert meizothrombin to thrombin instantaneously but that meizothrombin can persist in the prothrombinase complex long enough to interact with another macromolecule (i.e. HCII). Thrombin-HCII complexes are also observed in this type of experiment and may result, at least in part, from cleavage of the meizothrombin-HCII complex by prothrombinase as demonstrated in Fig. 6. Alternatively, some of the meizothrombin may be converted to thrombin before being inhibited by HCII. In this case, the resulting thrombin would appear to be inhibited by HCII fast enough to prevent the generation of meizothrombin(desF1) by cleavage of the thrombin-sensitive bond at Arg-155 and to prevent the appearance of thrombin amidolytic activity.

The function of HCII in vivo remains unknown. Although administration of exogenous dermaban sulfate to experimental animals produces a potent antithrombotic effect by activation of HCII in the circulation (34), humans with partial HCII deficiency do not appear to have an increased incidence of venous thromboembolic disease (35, 36). These observations support the hypothesis that HCII inhibits thrombin in the extravascular milieu (15), where it could modulate actions of thrombin in wound healing or inflammation. In the current study, we have demonstrated that the intermediates of prothrombin activation, meizothrombin and meizothrombin(desF1), can be inhibited rapidly by HCII in the presence of a glycosaminoglycan. Meizothrombin retains the N-terminal γ-carboxyglutamic acid domain of prothrombin and, therefore, the ability to bind to phospholipid membranes; it is unknown whether this intermediate can dissociate from the prothrombinase complex, interact with thrombomodulin, and activate protein C under physiologic conditions. Nevertheless, our data indicate that meizothrombin in the prothrombinase complex is susceptible to rapid inhibition by HCII. Meizothrombin(desF1), which lacks the N-terminal γ-carboxyglutamic acid domain, is perhaps more likely to diffuse away from the prothrombinase complex and activate protein C at a distant site. Under these circumstances, HCII could promote coagulation by inhibiting the activation of protein C by meizothrombin(desF1). The physiologic importance of the reactions described in this report could be established by demonstration of meizothrombin-HCII or meizothrombin(desF1)-HCII complexes in the circulation.

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