Activation-induced Exposure of the Thrombin Anion-binding Exosite

INTERACTIONS OF RECOMBINANT MUTANT PROTHROMBINS WITH THROMBOMODULIN AND A THROMBIN EXOSITE-SPECIFIC ANTIBODY*

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The activation of serine protease zymogens involves conformational changes that increase the affinity of substrate binding and the activity of the catalytic center. The activation of prothrombin is particularly complex and requires several cleavages in the proenzyme region in addition to the conserved activation cleavage after Arg$^{220}$. To understand how these cleavages lead to the exposure of the thrombin anion-binding exosite, a major macromolecular recognition site, interactions of recombinant human prothrombin derivatives with thrombomodulin, and an exosite-specific antibody were studied by competition binding and immunoprecipitation. By either method, the anion-binding exosite is not functional on prethrombin 2, which is cleaved after Arg$^{227}$ and lacks fragment 1-2, nor on meizothrombin, which is cleaved only after Arg$^{250}$. In contrast, the exosite is fully exposed on meizothrombin-des-F1, which is cleaved after both Arg$^{277}$ and Arg$^{286}$ and therefore lacks amino-terminal fragment 1 (F1). Thus, two events are required to create the exosite. First, cleavage after Arg$^{277}$ causes conformational changes that are much more extensive than those accompanying the activation of trypsinogen. Second, removal of amino-terminal F1 is necessary, perhaps to relieve steric hindrance. These results indicate that the F1 fragment regulates access to the thrombin exosite. The properties of meizothrombin-des-F1 suggest that this prothrombin derivative could have a biological function.

Conformational changes during zymogen activation are essential for most serine proteases to form high affinity complexes with substrate and for the enzyme-substrate complex to reach the transition state (1). In the case of trypsinogen, surface loops become reoriented after activation to trypsin, permitting the binding of substrate residues on the amino-terminal side of the scissile bond (2). For thrombin, however, the requirements for substrate recognition are more stringent. Thrombin function toward macromolecules requires both the serine active site and the anion-binding exosite that is some distance from the catalytic center (3). This exosite is essential for interactions with substrate residues on the carboxyl-terminal side of the scissile bond but is not available in prothrombin. Analogous interactions do not seem to be important for the activity of trypsin.

During blood coagulation, prothrombin is converted to thrombin by factor Xa that cleaves both the Arg$^{250}$-Ile$^{251}$ bond and the Arg$^{277}$-Thr$^{278}$ bond (Fig. 1) (4–11). (Numbering is from the amino terminus of human prothrombin) (12). Depending on the reaction conditions, either bond may be cleaved first. Cleavage after Arg$^{250}$ yields meizothrombin. This cleavage site is homologous to the activation cleavage sites of trypsinogen and other serine protease zymogens. Alternatively, cleavage after Arg$^{277}$ releases an amino-terminal polypeptide termed fragment 1-2 (F1-2) and yields the catalytically inactive prothrombin 2. Cleavage of both bonds by factor Xa yields active thrombin, which consists of two chains linked by a disulfide bond. In the presence of factor Va, calcium ions, and phospholipids, the pathway favored by factor Xa appears to be through meizothrombin to thrombin. The conversion of meizothrombin to thrombin needs not be direct, however, because meizothrombin and thrombin could cleave the Arg$^{155}$-Ser$^{156}$ bond to release fragment 1 (F1) and yield meizothrombin-des-F1.

The substrate specificity of the prothrombin intermediates illustrates the complexity of the thrombin activation mechanism. Meizothrombin and thrombin have similar activity toward small amide substrates, but human meizothrombin does not interact with macromolecules such as fibrinogen (13) and thrombomodulin (14). Cleavage of peptidyl amides requires the binding of substrate residues on the amino-terminal side of the scissile bond to appropriate subsites on the enzyme surface. These sites appear to be unavailable in prothrombin but are fully functional in meizothrombin. Removal from meizothrombin of F1-2 enables the exosite to bind substrate residues on the carboxyl-terminal side of the scissile bond. This is important for thrombin to efficiently cleave macromolecules in addition to small amides and esters.

To understand the mechanism by which proteolytic activation leads to the exposure of the thrombin exosite, we studied interactions of recombinant mutant prothrombins with thrombomodulin and a thrombin exosite-specific antibody. The results indicated that the exosite was not accessible in either meizothrombin or prethrombin 2 and that cleavage after either Arg$^{155}$ in meizothrombin or Arg$^{250}$ in prethrombin 2 was essential for interactions with several macromolecules. Thus, the conformational changes upon thrombin activation were much more extensive than those accompanying trypsin activation.

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1 The abbreviations used are: F1-2, prothrombin fragment 1-2; DIP, diisopropyl phosphoryl; F1, prothrombin fragment 1.
creating binding sites for substrate residues on both sides of the scissile bond. These results also suggest that the prothrombin F1 fragment regulates the accessibility of the thrombin anion-binding exosite.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human plasma thrombin (3200 NIH units/mg) was from Haematologic Technologies Inc., Essex Junction, VT. Human plasma prothrombin was provided by Drs. G. J. Broze and J. P. Miletich (Washington University). Human fibrinogen was from Kabivitrum, Stockholm, Sweden. Echis carinatus venom was from Sigma. Ecarin, the prothrombin activator from *E. carinatus* venom, was purified as described previously (14, 15). A rabbit anti-human thrombin antibody was provided by Dr. P. W. Majerus (Washington University). The polyclonal antibody recognized thrombin, prothrombin, and prothrombin activation intermediates and inhibited thrombin-clotting activities (16). Anti-thrombin autoantibody D from a patient with recurrent arterial thrombosis was purified by ammonium sulfate precipitation, Q-Sepharose ion-exchange chromatography, and protein G-Sepharose affinity chromatography, as described previously (17, 18). The purified IgG fractions inhibited thrombin-clotting activity with an *IC*<sub>50</sub> of 1.8 μM but had no effect on thrombin-antithrombin III interaction (17).

**Recombinant Prothrombins**—Expression and characterization of recombinant human wild type prothrombin and mutant prothrombins K52E<sup>7</sup>, R68E, R70E, and S205A were described previously (19). The active-site mutant prothrombin S205A had no detectable catalytic activities but bound to thrombomodulin normally. Plasmas pPTH66E and pPTK154E were constructed to express mutant prothrombins H66E and K154E, in which residues His 66 and Lys 154, respectively, of the human thrombin B chain were replaced by Glu. Plasmas pPTAΔE25 and pPTAΔQ23–25 were constructed to express mutant prothrombins E25 and Q23–25, in which residue Gln 25 and residues Pro 23, Gln 24, and Gln 25, respectively, of the human thrombin B chain were deleted. Mutant prothrombin H66E was stably expressed in CV-1 cells. Mutant prothrombins K154E, S205, and ΔQ23–25 were stably expressed in human embryonic kidney 293 cells.

**Expression and Purification of Recombinant Prethrombin 2**—Plasmid pΔPT was constructed to express human prethrombin 2 by deleting the sequences for the Gla and kringle domains of prothrombin. The codon for Val<sup>321</sup> of prothrombin was provided by Drs. G. J. Broze and J. P. Miletich (Washington University). The polyclonal antibody recognized thrombin, prothrombin, and prothrombin activated species fragment 1, fragment 2, thrombin A chain, and thrombin B chain, respectively. Selected derivatives of prothrombin are illustrated. Two pathways of prothrombin activation are indicated by *arrows* on the right side. Autoproteolytic cleavages of meizothrombin and meizothrombin des-F1 are indicated by *dashed arrows*.

**Results**

**Expression of Human Prethrombin 2**—Selected proteolytic derivatives of human prothrombin are illustrated schematically in Fig. 1. Previous studies have shown that human meizothrombin has normal activity toward small amides but very low activities for fibrinogen, platelets, and thrombomodulin (13, 14). Binding sites for these macromolecules are located in the thrombin anion-binding exosite (28), suggesting that a single cleavage after Arg<sup>230</sup> of prothrombin is not sufficient for the exposure of the thrombin exosite and that the presence of prothrombin fragment F1–2 may prevent access to the thrombin exosite. To test whether removal of F1–2 from prothrombin is sufficient to expose the exosite, we expressed recombinant human prethrombin 2 without a site-directed mutagenesis. The amino-terminal sequence of purified recombinant prethrombin 2 was determined to be Phe-Thr-Ala-Thr-Ser-Glu-Gln-Thr-Phe, corresponding to the amino-terminal sequence of purified recombinant prethrombin 2 by site-directed mutagenesis. The two ligands were sequentially used and had no effect on the residual thrombin B-chain. The first residue of the thrombin B-chain is Ile<sup>321</sup> of prothrombin.
of recombinant prethrombin 2 for human recombinant thrombomodulin was determined by competition equilibrium binding (Fig. 2). Prethrombin 2 did not inhibit significantly the binding of 125I-DIP-thrombin to thrombomodulin ($K_d > 100$ nM). However, when prethrombin 2 was activated by $E. carinatus$ venom, the resultant thrombin completely inhibited 125I-DIP-thrombin binding to thrombomodulin with a $K_d$ value of 6.3 ± 0.4 nM. This affinity is similar to that observed for plasma-derived thrombin or recombinant thrombin binding to recombinant human thrombomodulin (14, 19, 26). These results indicate that simply removing F1.2 from prothrombin is not sufficient to expose the thrombomodulin binding site and that additional cleavage of the peptide bond at Arg201-Ile321 is also required.

**Binding of Meizothrombin des-F1 to Thrombomodulin**—The binding of meizothrombin des-F1 to thrombomodulin was assessed by complex formation and immunoprecipitation (Fig. 3). [35S]Cysteine-labeled conditioned medium was prepared from control CV-1 cells or cells expressing active-site mutant prothrombin S205A (19). This active-site mutant allowed us to generate various stable prothrombin intermediates without further autoproteolytic cleavages (14). Meizothrombin S205A, meizothrombin des-F1 S205A, and thrombin S205A were immunoprecipitated with an anti-human thrombin polyclonal antibody (Fig. 3). Under nonreducing conditions, meizothrombin S205A exhibited a single band of 72 kDa (Fig. 3A, lane 2); under reducing conditions, it exhibited two bands of 50 and 32 kDa, corresponding to the prothrombin F1.2 plus the thrombin A chain and the thrombin B chain, respectively (Fig. 3B, lane 2). Meizothrombin des-F1 S205A migrated under nonreducing conditions as a species of ~54 kDa (Fig. 3A, lane 2), and it was reduced to the thrombin B chain and the small polypeptide consisting of F2 plus the thrombin A chain migrating near the dye front (Fig. 3B, lane 2).

When cell lysate containing thrombomodulin was added to the mixture of prothrombin intermediates, both meizothrombin des-F1 S205A and thrombin S205A, but not meizothrombin S205A, were co-precipitated by an anti-thrombomodulin antibody (Fig. 3, A and B, lanes 3). A nonspecific band was present in the reactions with or without thrombomodulin (Fig. 3, A and B, lanes 3 and 4). These results indicate that removal of the F1 fragment from meizothrombin enables meizothrombin des-F1 to bind to thrombomodulin, suggesting that the presence of the F1 fragment prevents access of the exosite in meizothrombin by macromolecules. This conclusion was further supported by the following studies with an anti-thrombin autoantibody.

**Localization of the Epitope for the Autoantibody D in the Thrombin Anion-binding Exosite**—In a previous report (17), an autoantibody (D) from a patient with recurrent arterial thrombosis was shown to inhibit the fibrinogen clotting and thrombomodulin binding activities of thrombin, suggesting that the antibody recognized an epitope in the thrombin exosite. To further localize the epitope for this autoantibody, the interaction of purified antibody D with various recombinant mutant thrombins was examined by immunoprecipitation (Fig. 4). Antibody D recognized wild type thrombin, mutant thrombins K52E and K154E, and active-site mutant thrombin S205A. In contrast, the antibody did not recognize mutant thrombins H66E, R68E, and R70E, nor deletion mutant thrombins ΔE25 and ΔPQE23–25. Residues His 66, Arg 68, Arg 70 and residues Pro 23, Gln 24, Glu 25 are located in two adjacent surface loops (loop segments Lys 66-Glu 76 and Phe 19-Leu 27, respectively) in the anion-binding exosite, whereas residues Lys 52 and Lys 154 are located in other surface loops near the exosite (29). As controls, the rabbit anti-human thrombin antibody (H) precipitated wild type thrombin and all mutant thrombins, whereas an antibody fraction isolated from normal subjects (N) did not immunoprecipitate recombinant thrombins (Fig. 4). Differences in the intensity of autoradiographic bands among the recombinant thrombins or thrombin derivatives correlated with the different levels of prothrombin expressed in the stable cell lines. These results indicate that antibody D binds to a region in the thrombin exosite that overlaps the previously identified thrombomodulin binding site (19). The conclusion was further supported by separate fibrinogen clotting assays in which mutant thrombin R70E was completely resistant to the inhibition of antibody D at concentrations up to 150 μg/ml, whereas plasma-derived thrombin was inhibited in a dose-dependent manner (data not shown). These results are consistent with the previous finding that antibody D inhibits thrombin interaction with fibrinogen and thrombomodulin but not thrombin amidolytic activity (17).

**Binding of Antibody D to Prothrombin and Prethrombin 2**—Antibody D was employed to detect conformational changes of the thrombin exosite during prothrombin activation. Neither
prothrombin S205A, as described under "Experimental Procedures." After activation with E. carinatus venom, recombinant thrombins were immunoprecipitated with either an anti-thrombin polyclonal antibody (H) or autoantibody D (D). Proteins were analyzed on 10% polyacrylamide gels under nonreducing (panel A) or reducing (panel B) conditions.

**FIG. 5. Immunoprecipitation of prothrombin, thrombin, and thrombin by autoantibody D.** [35S]Cysteine-labeled conditioned media were prepared from control CV-1 cells or cells expressing human recombinant prothrombin (rPT), prothrombin mutants K52E, H66E, R68E, R70E, K154E, and S205A, prothrombin deletion mutants ΔE25, and ΔPQE23-25, as described under "Experimental Procedures." Proteins were immunoprecipitated with either an anti-thrombin polyclonal antibody (H), autoantibody D (D), or a negative control IgG fraction from normal individuals (N). Proteins were analyzed on 10% polyacrylamide gels under reducing conditions.

**FIG. 6. Immunoprecipitation of meizothrombin and meizothrombin des-F1 by autoantibody D.** [35S]Cysteine-labeled conditioned media were prepared from CV-1 cells expressing recombinant prothrombin active-site mutant S205A. Conditioned medium was either treated with crude E. carinatus venom (Venom) or purified prothrombin activator ecarin (Ecarin). Prothrombin derivatives were immunoprecipitated with either an anti-thrombin polyclonal antibody (H) or autoantibody D (D). Proteins were analyzed on 10% polyacrylamide gels under nonreducing (panel A) or reducing (panel B) conditions.

recombinant wild type prothrombin (Fig. 5, lane 5) nor prothrombin 2 (Fig. 5, lane 11) was immunoprecipitated by antibody D. After activation with E. carinatus venom, however, the resultant thrombin forms were precipitated (Fig. 5, lanes 8 and 14). These results indicate that the thrombin anion-binding exosite is not available to antibody D in prothrombin 2. This conclusion is consistent with the inability of recombinant prethrombin 2 to bind thrombomodulin (Fig. 2).

**Binding of Antibody D to Meizothrombin and Meizothrombin des-F1**—The interaction of antibody D with meizothrombin and meizothrombin des-F1 was examined by immunoprecipitation with active-site mutant prothrombin S205A. [35S]Cysteine-labeled prothrombin S205A in conditioned medium was treated either with crude E. carinatus venom or purified ecarin. Ecarin cleaved the single peptide bond at Arg155-Ile321 of prothrombin (15) and yielded meizothrombin S205A (14). Crude E. carinatus venom variably cleaved in addition the Arg155-Ser156 and Arg271-Thr272 bonds to generate meizothrombin des-F1 S205A and thrombin S205A. All of these forms were immunoprecipitated efficiently by rabbit polyclonal antibody H (Fig. 6, A and B, lanes 1 and 3).

Thrombin S205A was recognized by antibody D (Fig. 4; Fig. 6A, lane 2), indicating that mutation of the catalytic serine did not affect the binding of the antibody to the thrombin exosite. In contrast, antibody D bound poorly to meizothrombin S205A (Fig. 6, A and B, lanes 4). As measured by densitometry, no more than 20% of meizothrombin S205A was precipitated by antibody D compared with that precipitated by polyclonal antibody H (Fig. 6A, lanes 3 and 4). The difference was more dramatic in the reducing gel (Fig. 6B, lanes 3 and 4). This suggests that in meizothrombin the epitope for antibody D in the exosite is not fully exposed. This is consistent with our previous observations that human meizothrombin has very low affinity for thrombomodulin (14). Interestingly, meizothrombin des-F1 S205A was precipitated with similar efficiency by antibody D and by antibody H (Fig. 6, A and B, lanes 1 and 2), suggesting that the thrombin exosite became further exposed upon the removal of the F1 fragment from meizothrombin. These results are in agreement with the finding that meizothrombin des-F1 can bind to thrombomodulin (Fig. 3).

**DISCUSSION**

Thrombin is a remarkable serine protease that plays an important role in a variety of biological events. Thrombin-specific function is determined by its unique structural features. These include several enlarged insertion surface loops, which restrict the access of substrates to the serine active center, and clusters of positively charged residues in the anion-binding exosite, which constitutes a major substrate recognition site. A number of studies have demonstrated that the exosite contributes significantly to thrombin interactions with fibrinogen, thrombomodulin, heparin cofactor II, the thrombin platelet receptor,
and hirudin (28). In this report, we used recombinant prothrombins to study conformational changes of the thrombin exosite induced during prothrombin activation and to understand how activation cleavages convert prothrombin to an active enzyme.

Reorientation of surface loops with substrate binding function is an important step in zymogen activation. Our results indicate that the conformational changes upon thrombin activation are much more extensive than those accompanying trypsin activation. The cleavage of an activation hexapeptide from trypsigen exposes a new Ile-Val amino terminus and enables trypsin to interact with substrates. Structures determined by x-ray crystallography have shown major conformational changes in the trypsin surface loops that interact with substrate residues on the amino-terminal side of the scissile bond (2). Similar conformational changes in corresponding thrombin surface loops appear to be created by the analogous cleavage after Arg220 in prothrombin, because meizothrombin is able to bind small amide substrates (13) and a thrombin active-site inhibitor, N-[(3-ethyl-1,5-pentanediyl)amido]lysine (30). Interactions with these peptide amides depend on subsites of thrombin that bind substrate residues on the amino-terminal side of the scissile bond. In addition, the affinity of meizothrombin for bovine thrombin (35) and human meizothrombin and that an additional cleavage at the carboxyl-terminal side of the scissile bond that interact with these macromolecules (Figs. 3 and 6). Although the kringle domains of prothrombin are generally considered to be important for interactions within the prothrombinase complex (33), the precise function of each kringle domain remains unknown. Studies with recombinant mutant prothrombins lacking kringle domains have indicated that these kringle domains are not necessary for thrombin binding to phospholipid membranes (34). We have shown that the presence of the F1 fragment prevents access to the thrombin exosite by macromolecules, thereby inhibiting the function of human meizothrombin. This may represent a regulatory function of kringle domains.

In this study, human recombinant prethrombin 2 was expressed and purified. As determined by competition binding studies, the affinity of prethrombin 2 for human recombinant thrombomodulin was at least 20-fold lower than that of bovine thrombin (35). Studies with autoantibody D demonstrated that the exosite was not accessible in human recombinant prethrombin 2 (Fig. 5). It has been reported, however, that hirugrin binds to bovine thrombin and prethrombin 2 with similar affinity (36). Hirugrin (37) is a synthetic dodecapetide corresponding to the carboxyl-terminal portion of hirugrin, which interacts with the thrombin exosite (38, 39). It is possible that relatively small size and greater flexibility enable hirugrin to bind to regions on prethrombin 2 that are not accessible to macromolecules such as thrombomodulin.

The binding of meizothrombin des-F1 to thrombomodulin (Fig. 3) suggests that this complex could have functional significance. The cleavage site at Arg155-Ser156 between the F1 and F2 fragments is conserved among prothrombins of all species thus far studied (12, 40-42), suggesting evolutionary pressure to maintain this prothrombin intermediate. In addition, the 3-dimensional structure of a noncovalent F2-thrombin complex shows that F2 interacts with the putative heparin binding site near the carboxyl-terminal a-helix of thrombin (43). Meizothrombin des-F1 may, therefore, be resistant to heparin inhibition. In a previous study, bovine meizothrombin des-F1 was reported to catalyze protein C activation in a thrombomodulin-dependent manner (44). Further studies are required to test the stability of the human meizothrombin des-F1-thrombomodulin complex and to determine if the complex has an unexpected substrate specificity.

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