Thrombin acts on many protein substrates during the hemostatic process. Its specificity for these substrates is modulated through interactions at regions remote from the active site of the thrombin molecule, designated exosites. Exosite interactions can be with the substrate, cofactors such as thrombomodulin, or fragments from prothrombin. The relative activity of α-thrombin for fibrinogen is 10 times greater than that for protein C. However, the relative activity of meizothrombin for protein C is 14 times greater than that for fibrinogen. Modulation of thrombin specificity is linked to its Na⁺-binding site and residues in autolytic loop-2 that interact with the Na⁺-binding site. Recombinant prothrombins that yield recombinant meizothrombin (rMT) and rMT des-fragment 1 (rMT(desF1)) enable comparisons of the effects of mutations at the Na⁺-binding site (Asp554) and deletion of loop-2 (Glu466-Thr469) on the relative activity of meizothrombin for several substrates. Hydrolysis of β-butyryloxycarbonyl-VPRP-p-nitroanilide by α-thrombin, recombinant α-thrombin, or rMT(desF1) was almost identical, but that by rMT was only 40% of that by α-thrombin. Clotting of fibrinogen by rMT and rMT(desF1) was 12–16% of that by α-thrombin, as already known. Strikingly, however, although meizothrombins modified by substitution of Asp554 with either Ala or Leu or by deletion of loop-2 had 6–8 and <1%, respectively, of the clotting activity of α-thrombin, the activity of these meizothrombins for protein C was increased to >10 times that of α-thrombin. It is proposed that interactions within thrombin that involve autolytic loop-2 and the Na⁺-binding site primarily enhance thrombin action on fibrinogen, but impair thrombin action on protein C.

In the procoagulant reactions of blood coagulation, α-thrombin plays a pivotal role in activating coagulation factors (factors V, VIII, XI, and XIII and fibrinogen) and in stimulating a variety of cells such as platelets, leukocytes, and endothelial cells (1–7). In anticoagulant reactions, α-thrombin binds to thrombomodulin, and the thrombin-thrombomodulin complex activates protein C, the proteinase that is key to shutting down the procoagulant processes (8). Meizothrombin and meizothrombin des-fragment 1 are short-lived intermediates in the activation process of prothrombin to α-thrombin; both have enzymatic activity (9–17). Fig. 1 illustrates the cleavage sites in prothrombin and the structures of α-thrombin, meizothrombin, and meizothrombin des-fragment 1. Meizothrombin in its normal interaction with thrombomodulin efficiently activates protein C in vitro, but it cleaves fibrinogen inefficiently (18). Moreover, meizothrombin exhibits only 2% of the platelet aggregation activity of thrombin, and its rate of inhibition by antithrombin III is 43% if that of α-thrombin (18, 19).

In the α-thrombin molecule, both autolytic loop-2 (Glu146–Lys149-β-Lys148-β-Leu147-β-Lys146-β) and the Na⁺-binding region (Asp221–Tyr225-β-Leu224-β-Leu223-β-Leu222-β) are involved in determining thrombin specificity for fibrinogen clotting and protein C (20–24). When these regions are deleted or substituted in α-thrombin to create a non-Na⁺-binding thrombin form, fibrinogen-clotting activity is remarkably low, but the protein C activator activity decreases only slightly (22–24). No systematic examination of the role of the Na⁺-binding region and autolytic loop-2 in meizothrombin has been made. If mutations in these regions are constructed in meizothrombin, investigation of the relative activities of the mutants will enable the relationships between Na⁺ binding and the functional differences between α-thrombin and meizothrombin to be determined. Determination of the specificity differences between α-thrombin and meizothrombin is made difficult by the autolytic cleavages that occur in prothrombin and meizothrombin. We have constructed recombinant prothrombin molecules with substitutions of Ala for Arg at the sites of autolysis to eliminate the confounding cleavages, as reported previously (18), that permit definitive testing of their specificity for two substrates, protein C and fibrinogen.

We then constructed novel meizothrombin mutants with alterations in the Na⁺-binding site or with a deletion of autolytic loop-2. We report here that these loop-2- and Na⁺-binding site-modified recombinant meizothrombin derivatives show moderate to large reductions in fibrinogen-clotting activity, but large enhancement of protein C activator activity.

**EXPERIMENTAL PROCEDURES**

Materials—Human prothrombin was purified following a published method (25). Human protein C was purified using the following modifications of the prothrombin purification method. The Ba²⁺-protein precipitate, after (NH₄)₂SO₄ precipitation (25), was applied to a DEAE-Sepharose column and eluted with a linear gradient of 0–0.5 M NaCl. Fractions that contained factors IX and X and protein C were applied to heparin-Sepharose and eluted with 0.25 M NaCl. The protein C-containing fractions were applied to a Mono Q column and eluted with a linear gradient of 0–0.5 M NaCl. The purification of human protein C was analyzed by SDS-PAGE at each step. The thrombin activator ecarin...
from *Echis carinatus* venom was purified as reported previously (26, 27). Human recombinant thrombomodulin (rTM)2 was a kind gift from Dr. Yasuo Sasaki (Asahi Kasei Corp., Shizuoka, Japan). The chromogenic substrate for thrombin, t-butoxycarbonyl-Val-Pro-Arg-p-nitroanilide (Boc-VPR-pNA), and the substrate for activated protein C, Boc-Leu-Ser-Thr-Arg-4-methylcoumaryl-7-amide (Boc-LSTR-MCA), were from Seikagaku Corp. (Tokyo, Japan) and Peptide Institute, Inc. (Osaka, Japan), respectively.

**Construction of Prothrombin Mutants**—A full-length human prothrombin cDNA was isolated from a human liver cDNA library using two oligonucleotide primers from human prothrombin to which restriction enzyme sites (underlined in the following sequences) were added. The 5′-primer sequence was 5′-TTTTAGATTCACAGCGGCCGCACTGC- CG-3′, and the 3′-primer sequence was 5′-CATGATTGGCTATGACAAC- GAGACCGGCCGCAGGTTC-3′. An amplified cDNA of 1.9 kb was cloned into pUC19, and the resulting pUCpHT was sequenced using a DSEQ-2000L DNA sequencer (Shimadzu Corp., Kyoto, Japan). pUCpHT was digested with EcoRI and NotI, and the 1.9-kb cDNA fragment was separated by agarose gel electrophoresis and purified using a QIAquick DNA purification kit (QIAGEN GmbH, Hilden, Germany). This fragment was cloned into a mammalian expression vector (pSecTag, Invitrogen) that contains Myc and His tags at the C-terminal end. The protein product of this construct, i.e. human prothrombin produced with pSecTag/pHT, is designated PT-tag. Prothrombin mutants were constructed from pUCpHT by a PCR-based site-directed mutagenesis method (28). The sequences of all prothrombin mutants were determined by DNA sequencing. In the mutant PT-RA155, Arg155 is replaced by Ala (the site between fragments 1 and 2) (see Fig. 1); and in the mutant PT-RA271/284, Arg271 and Arg284 are replaced by Ala (see Fig. 1). In the mutant PT-RA271/284, Arg271, Arg284, and Arg342 are replaced by Ala (29). The Nα-binding site mutants PT-DAS554 and PT-DL554 have Arg354 replaced by Ala or Leu, respectively. In PTΔ466–469, Glu466-Thr469 were deleted from PT-RA155/271/284. All PT constructs contain Myc and His tag sequences at their C-terminal ends, adding 3.4 kDa to these proteins compared with their hypothetical plasma prothrombin-derived homologs. The Myc tag was used to detect the cloned products by anti-Myc immunoblotting.

**Expression of Recombinant Prothrombin Mutants**—Expression constructs were transfected into COS-7 cells using FuGENE 6 (Roche Molecular Biochemicals). The transfected cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum for 24 h. Two cultures were grown: one with 10 μg/ml vitamin K and one without. The transfected cells were subsequently transferred to serum-free Dulbecco’s modified Eagle’s medium, again either with or without 10 μg/ml vitamin K, and further incubated for 48 h. Expressed PT-tag secreted into the culture medium was recovered after centrifugation at 2000 × g for 10 min at 4 °C to remove the cells.

The supernatant culture medium was applied to an Nε-nitrotriacetic acid (Nε-NTA) column (8-ml volume; QIAEXIN GmbH) equilibrated with 50 mM HEPES (pH 7.5), 0.5 M NaCl, 0.1% protease inhibitor cocktail (Sigma), and 0.5 mM Nε-NTA to capture the His-tagged protein. The effluent was directly connected to a reverse-phase column (ProBlottTM, PerkinElmer Life Sciences), and stained with 0.1% Amido Black 10B in 50% methanol and 10% acetic acid. Protein bands were sequenced by nanofocusing on 10% SDS-polyacrylamide gels, transferred to a polyvinyldene difluoride membrane (ProBlottTM, PerkinElmer Life Sciences), and stained with 0.1% Amido Black 10B in 50% methanol and 10% acetic acid. Protein bands to be sequenced were cut out and washed with deionized water (MilliQ, Millipore Corp.). The amino acid sequences were determined with an Applied Biosystems Model 477A-120A amino acid sequencer.

**Amino Acid Sequence Determination**—N-terminal amino acid sequences of the recombinant prothrombins were determined in Nε-NTA-nitrotriacetic acid-purified fractions. Aliquots of the column fractions were diluted in 2× SDS buffer subjected to electrophoresis on 10% SDS-polyacrylamide gels, transferred to a polyvinyldene difluoride membrane (ProBlottTM, PerkinElmer Life Sciences), and stained with 0.1% Amido Black 10B in 50% methanol and 10% acetic acid. Protein bands to be sequenced were cut out and washed with deionized water (MilliQ, Millipore Corp.). The amino acid sequences were determined with an Applied Biosystems Model 477A-120A amino acid sequencer.

**Protein C Activator Activity**—Plasma or recombinant prothrombin (2 nm final concentration) was activated by ecarin (0.2 nm final concentration) in TBS and 0.1% bovine serum albumin at 37 °C in a 96-well microtiter plate. After 30 min, equal volumes of human protein C (80 nm final concentration), rTM (either 40 nm or 200 or various concentrations) or 3.1 (mM/liter) phosphatidylycholine/phosphatidylserine vesicles (100 μm final or various concentrations) in TBS containing 0.1% bovine serum albumin were added to the reaction mixture and incubated for 60 min at 37 °C to activate protein C. Activated protein C was determined from the initial rate of hydrolysis of Boc-LSTR-MCA (1 mM final concentration), measured by 7-aminocoumarin liberation (λex = 380 nm, λem = 465 nm) with a GENios fluorometer (Tecan).

**Platelet Aggregation Assay**—Platelet aggregation was measured by determining the changes in light transmission of platelet suspensions with an optical aggregometer (Niko Bioscence Hema Trace 610). Maximal aggregation induced by 100 nM r-α-thrombin was defined as 100%. Washed platelets were prepared from blood by healthy volunteers with informed consent. All volunteers denied taking any medications that would alter the response of platelets to thrombin. Blood was collected into 0.1 volume of 3.8% sodium citrate by venipuncture and incubated at 37 °C for 30 min at room temperature to allow platelet-rich plasma to separate, which were then washed twice with 15%/acid/citrate/dextrose and 100 μl prostacyclin. Platelet pellets were suspended in HEPES/Tyrode’s buffer (20 mM HEPES (pH 7.4), 138 mM NaCl, 3.3 mM NaHPO4, 2.9 mM KCl, 1.0 mM MgCl2, 1.0 mM CaCl2, and 1 mg/ml glucose). Resuspended platelets were incubated for 2 min at 37 °C, after which r-α-thrombin, rMT, rMT-DL554, or rMTΔ466–469 (0.1–100 nm final concentrations) was added to initiate aggregation.

**RESULTS**

To compare the specificity of meizothrombin and meizothrombin des-fragment 1 with that of α-thrombin, recombinant prothrombin molecules with a substitution of Ala for Arg at the sites of autolysis were constructed. Fig. 1 shows the cleavage sites and the sites of the mutations; the substitutions and deletions are defined in Table I. PT-tag is an recombinant prothrombin with Myc and His tags at the C terminus, but without other mutations. PT-tag thus serves as the control for recombinant prothrombin and r-α-thrombin. PT-RA155, with Arg155 substituted by Ala, is not cleaved to form fragments 1 and 2, but produces r-α-thrombin that is structurally identical to that derived from PT-tag. PT-RA271/284 and PT-RA155/271/284 produce rMT(desF1) and rMT, respectively, and have been...
reported previously (29). The three mutants PT-DA554, PT-DL554, and PT/H9004–469 are new constructs designed to produce rMT-DA554, rMT-DL554, and rMT/H9004–469, respectively. These products permit the evaluation of the contributions of the Na\(^+\)-binding site Asp residue and autolytic loop-2 to the specificity of meizothrombins for fibrinogen and protein C.

Expression in COS Cells and Purification of Recombinant Prothrombins—The seven recombinant prothrombin constructs were transfected into COS cells. All prothrombin mutants were efficiently expressed (Fig. 2A). The importance of Na\(^+\)-carboxylation for prothrombin secretion by COS cells was also demonstrated. Na\(^+\)-carboxylation, which modifies a glutamate in the Na\(^+\)-carboxyglutamic acid domain, depends on vitamin K as a cofactor. Recombinant prothrombin derivatives in COS cells were efficiently secreted into the culture medium only in the presence of vitamin K (Fig. 2B). Thus, prothrombin secretion in this cell system depends on Na\(^+\)-carboxylation.

The mutants PT-tag and PT-RA271/284 had the molecular masses expected for wild-type prothrombin plus the mass of the tag (Fig. 2A and B). For recombinant prothrombins with the Arg\(^{155}\)-to-Ala mutation, the molecular masses appeared higher upon SDS-PAGE by \(3-4\) kDa (Fig. 2A). The N-terminal amino acid sequences of all expressed recombinant prothrombins were identical to that of wild-type prothrombin (Ala-Asn-Thr-Phe-Leu-). All mutants bound to an Ni\(^2+\)-nitrilotriacetic acid column (Ni\(^2+\)-chelating bead), implying that the His tag at the C terminus was correctly constructed and intact. Because both the N and C termini appeared to be correct, the mobility shift of the Arg155 mutants was not investigated further.

The prothrombin precursor mutants with Myc and His tag sequences at their C termini were purified using metal-chelating column chromatography and detected by anti-Myc antibody. The purity of the PT-tag-containing fractions eluted from the column was demonstrated by SDS-PAGE and by immunoblot analysis (Fig. 3). No autolysis products could be detected in the PT-tag-containing fractions. All mutants were purified by identical metal-chelating column chromatographic procedures (data not shown).

### Table I

| Precursor                 | Thrombin/FXa\(^a\) cleavage sites | Loop-2\(^b\) | Na\(^+\)-binding site | Activated forms |
|---------------------------|-----------------------------------|--------------|------------------------|----------------|
| Plasma prothrombin        | Arg\(^{155}\) Arg\(^{271}\) Arg\(^{284}\) | Glu\(^{466}\)-Thr\(^{469}\) | Asp\(^{554}\) c | α-Thrombin           |
| PT-tag                    | Arg\(^{155}\) Arg\(^{271}\) Arg\(^{284}\) | Glu\(^{466}\)-Thr\(^{469}\) | Asp\(^{554}\) c | r-Thrombin           |
| PT-RA155                  | Ala\(^{155}\) Arg\(^{271}\) Arg\(^{284}\) | Glu\(^{466}\)-Thr\(^{469}\) | Asp\(^{554}\) c | r-MT(desF1)         |
| PT-RA271/284              | Arg\(^{155}\) Ala\(^{271}\) Ala\(^{284}\) | Glu\(^{466}\)-Thr\(^{469}\) | Asp\(^{554}\) c | rMT               |
| PT-RA155/271/284          | Ala\(^{155}\) Ala\(^{271}\) Ala\(^{284}\) | Glu\(^{466}\)-Thr\(^{469}\) | Asp\(^{554}\) c | rMT-DA554          |
| PT-DL554                  | Ala\(^{155}\) Ala\(^{271}\) Ala\(^{284}\) | Glu\(^{466}\)-Thr\(^{469}\) | Asp\(^{554}\) c | rMT-DL554          |
| PT-DL554                  | Ala\(^{155}\) Ala\(^{271}\) Ala\(^{284}\) | Glu\(^{466}\)-Thr\(^{469}\) | Asp\(^{554}\) c | rMT\(^{466}-469\) |

\(^a\) Factor Xa.

\(^b\) Autolytic loop-2 (Glu\(^{146}\)-Lys\(^{149}\), chymotrypsin numbering).

\(^c\) Asp\(^{222}\) (chymotrypsin numbering).
Activation of Prothrombin Mutants and Functional Characterization of Activated Forms—As anticipated, PT-tag and PT-RA155 were readily converted to r-\(\alpha\)-thrombin by the prothrombin activator ecarin, whereas PT-RA271/284 and PT-RA155/271/284 were activated to rMT(desF1) and rMT, respectively (Fig. 4). PT\(\Delta\)466–469, PT-DA554, and PT-DL554 were similarly converted to the mutant meizothrombin derivatives. All recombinant prothrombins were rapidly activated to their cleaved forms by ecarin, implying that the structure around the relevant scissile bond, Arg320, was not grossly perturbed.

We assessed the activity of each recombinant thrombin and meizothrombin derivative by measuring Boc-VPR-pNA hydrolysis rates and by fibrinogen clotting (Table II). PT-RA155 and r-\(\alpha\)-thrombin produced from PT-tag had activities for both substrates experimentally indistinguishable from those of \(\alpha\)-thrombin derived from native prothrombin. The Boc-VPR-pNA hydrolytic activity of rMT(desF1) was 94% of that of r-\(\alpha\)-thrombin, but rMT activity was decreased to 38%. This is consistent with a published report that the prothrombin fragments, which remain covalently linked in rMT, reduce \(\alpha\)-thrombin hydrolysis of peptide pNA (30). rMT-DA554 and rMT-DL554 had 8 and 6% of the r-\(\alpha\)-thrombin activity, respectively. rMT\(\Delta\)466–469 had no significant fibrinogen-clotting activity (<1%). Thus, deletion of autolytic loop-2 abolishes meizothrombin activity for fibrinogen, and mutation of the Na\(^+\)-binding residue reduces this activity.

Protein C Activator Activity—We examined each of the forms of thrombin and meizothrombin for their ability to activate protein C in the presence of phospholipids and thrombomodulin (Fig. 6A). The significance of \(\gamma\)-carboxyglutamic acid domain-containing fragment 1 can be assessed by comparing the activity of rMT(desF1), which lacks fragment 1, with that of the fragment 1-containing rMT derivatives (Fig. 6A, black bars). The activities of rMT and its derivatives were dramatically higher for protein C than those of both rMT(desF1) and r-\(\alpha\)-thrombin, in contrast to the lower activities of rMT and its derivatives for fibrinogen. In the absence of covalently attached fragment 1, no enhancement of protein C activation was observed, i.e. with rMT(desF1) or r-\(\alpha\)-thrombin (Fig. 6A).
and C) shows the effects of rTM and phospholipid concentration on protein C activation by r-α-thrombin, rMT, and rMT derivatives and verifies that the observations are not unique to a limited set of reactant concentrations.

Platelet Aggregation Activity—α-Thrombin is a potent physiological platelet activator. The effects of r-α-thrombin and rMT derivatives on platelet aggregation are shown in Fig. 7. Whereas r-α-thrombin at 3 nM induced 95% platelet aggregation, rMT at 100 nM induced only ~60% aggregation. Both rMT-DL554 and rMTΔ466–469 had poor platelet aggregation-inducing activity (<5%), even at the highest concentration tested (100 nM). This result shows that both meizothrombin derivatives rMT-DL554 and rMTΔ466–469 lack significant platelet aggregation-inducing activity. Ecarin, the snake venom-derived prothrombin activator present in these reaction mixtures, did not initiate platelet aggregation (data not shown).

**Table II**

| Activated forms | Chromogenic activity | Fibrinogen-clotting activity (P) | Protein C activator activity (P) | RAPa |
|-----------------|----------------------|---------------------------------|----------------------------------|------|
| α-Thrombin      | 20.0 (100%)          | 11.1 (98.2%)                    | 23.1 (118%)                      | 1.2  |
| r-α-thrombin    | Derived from PT-tag  | 20.0 (100%)                     | 11.3 (100%)                      | 19.6 (100%) |
| Derived from PT-RA155 | 19.9 (99.5%) | NDa                              | ND                              | ND   |
| rMT             | 18.8 (94.0%)         | 1.75 (15.5%)                    | 24.9 (127%)                      | 8.2  |
| rMTΔdesF1       | 7.7 (38.5%)          | 1.30 (11.5%)                    | 275.9 (1410%)                    | 123  |
| rMT-DL554       | 4.3 (21.5%)          | 0.93 (8.2%)                     | 225.7 (1150%)                    | 140  |
| rMTΔ466–469     | 4.3 (21.5%)          | 0.70 (6.2%)                     | 319.1 (1630%)                    | 263  |
| rMTΔ466–469     | 4.4 (22.0%)          | <0.11 (<1.0%)                  | 314.7 (1610%)                    | >1610|

Relative anticoagulant potency of a mutant, PiF, where P and F are the rates for protein C activator and fibrinogen-clotting activities, respectively. Maximal activities given by r-α-thrombin are defined as 100%.

APC, activated protein C; ND, not determined.

**DISCUSSION**

Meizothrombin is known to have enhanced protein C activator activity and diminished fibrinogen-clotting activity compared with α-thrombin (11, 18, 32). In fibrinogen clotting, prothrombin fragment 2 in meizothrombin and meizothrombin(desF1) inhibits interaction with fibrinogen (32). The marked stimulation of meizothrombin protein C activator activity by phospholipids clearly indicates the importance of fragment 1, which includes the Ca2+- and phospholipid-binding γ-carboxyglutamic acid domain (33, 34), for enhancement of the reaction by phospholipid vesicles (18). Côté et al. (18) observed no enhancement of meizothrombin activity for protein C when soluble thrombomodulin was used rather than the lipid-binding native form of thrombomodulin. One plausible explanation for this difference is the contribution that binding to phospholipid membranes makes to the reaction rates by promoting the formation of thrombin- or meizothrombin-thrombomodulin complexes. More complex alterations in the conformation of meizothrombin can also be speculated to occur, e.g. as a result of an intramolecular interaction involving the fragment 1 region and a thrombin exosite. Such conformational changes might be similar to the conformational changes that are caused by thrombomodulin in α-thrombin that convert it from a fibrinogen-preferring to a protein C-preferring protease.
Protein C activation was assayed by liberation of 7-amino-4-methylcou-thrombin and meizothrombin derivatives. Receptor thrombin activity to r-thrombin; rMT, thrombin from wild-type pro-thrombin; rMT(desF1); rMT; rMT-DA554; rMT-DL554; rMT-DL566-469. C, effect of phospholipid on activation of protein C. Various concentrations of phospholipid were tested in the presence of a fixed rTM concentration (40 nM). Symbols are as defined for B. PCPS, 3.1 (mol/mol) phosphatidylcholine/phosphatidylserine vesicles.

In α-thrombin, deletion of autolytic loop-2 (Glu146–Lys149E; chymotrypsin numbering; Glu466–Lys474 of prothrombin) or the substitution of the Na+–binding site residues (Asp221–Tyr225, chymotrypsin numbering; Asp552–Tyr557 of prothrombin) decreases its fibrinogen-clotting activity, but affects only slightly its protein C activator activity in the presence of thrombomodulin and phospholipids (20, 23, 35). In particular, deletion of the entire loop-2 of α-thrombin nearly eliminates clotting activity, but protein C activator activity is reduced by only 2-fold (23). Most of the effect must originate from direct perturbation of fibrinogen binding due to the loss of critical interactions between loop-2 and fibrinogen in the autolytic loop-2 deletion mutant.

In this study, Ala or Leu substitution at the Na+–binding site (Asp554) or deletion of autolytic loop-2 residues (Glu466–Thr469) (Fig. 1) in meizothrombin was used to test the importance of the Na+–binding site and autolytic loop-2, known “specificity determinants” for α-thrombin, in meizothrombins. The results demonstrate that these regions are also specificity determinants for meizothrombin, but with opposite effects in meizothrombin and α-thrombin. Whereas autolytic loop-2 and the Na+–binding residue Asp554 are crucial for α-thrombin action on fibrinogen, mutation of the Na+–binding site in meizothrombin causes only a modest decrease in action on fibrinogen.

The differences in the relative specific activity of the activated meizothrombin mutants for fibrinogen and protein C are more dramatic; in fact, they change from reduced to enhanced relative activity for these two substrates. Specifically, in fibrinogen clotting, rMT (desF1) to 16% relative to r-α-thrombin (Fig. 5 and Table II). rMT-DA554 (8%) and rMT-DL554 (6%) had even lower clotting activity. Because these rMT derivatives (rMT-DA554, rMT-DA554, and rMT-DL566-469) have no Na+–binding site, their conformation is possibly similar to the non-Na+–binding form of thrombin (35).

The control mutants (r-α-thrombin from PT-tag and rMT-(desF1)) were indistinguishable from native α-thrombin in their ability to catalyze protein C activation in the presence of thrombomodulin and phospholipid vesicles (Fig. 6A). The pro-
Mutations That Enhance Protein C Activation by Meizothrombin

Protein C activator activities of both rMT and its derivatives were increased by 10-fold or more compared with those of α-thrombin (Fig. 6A). These four highly active proteins are distinguished by having the fragment 1 region attached, whereas α-thrombin, r-α-thrombin, and rMT(desF1) do not. Both rTM and phospholipids increased the protein C activator activities of the meizothrombin mutants with fragment 1 (Fig. 6, B and C). Interestingly, even in the absence of thrombomodulin, the fragment 1-containing proteins showed enhanced protein C activator activity compared with α-thrombin (Fig. 6A). Table II summarizes the relative chromogenic, fibrinogen-clotting, and protein C activator activities of the activated forms of recombinant prothrombins. Most significant is the fact that, although rMT-DA554, rMT-DL554, and rMTA466–469 lack an Na⁺-binding function, these activated recombinant meizothrombins have very high protein C activator activities, indicating that the Na⁺-binding site has an insignificant role (if any) in protein C activation by meizothrombin.

The other procoagulant process recognized as a key function of α-thrombin is activation of platelets. In this process, similar to the action of these various forms of thrombin on fibrinogen, the mutations resulted in reduction in relative activity. Fig. 7 shows the effects of r-α-thrombin, rMT, and meizothrombin derivatives on the aggregation of washed platelets. Both rMT-DL554 and rMTA466–469 had essentially no platelet aggregation activity. These results imply that rMTA466–469 has little or no protease activities for fibrinogen or a physiological substrate such as PAR-1 (protease-activated receptor-1), the thrombin receptor on platelets. Further studies are required to determine whether this is due to decreased binding or decreased ability to catalyze proteolytic cleavage.

It is useful to attempt to relate these observations to the crystal structure of thrombin, although it must be noted that surface residues can be subject to crystal packing variations that prevent detailed interpretations from being made. Fig. 8A shows a model of α-thrombin derived from one of the thrombin crystal structures (36). Active-site residues are shown in red. The Na⁺-binding site (Asp524–Asp554) and the autolytic loop-2 region (Glu466–Thr469) are yellow and orange, respectively. Other side chains of amino acids in dark green are the interaction sites of fibrinogen, which are located throughout the areas surrounding both the Na⁺-binding site and the loop-2 region (24, 37–39). rMTA554, rMT-DL554, and rMTA466–469 lack a functional Na⁺-binding site in the thrombin region shown in Fig. 8A. Moreover, rMTA466–469, which is defective in autolytic loop-2, is missing Glu466 (Glu446, chymotrypsin numbering), which ion pairs with Arg253 (Arg231A, chymotrypsin numbering) of the Na⁺-binding site (31, 40, 41), and had essentially no fibrinogen-clotting activity, suggesting that the side chains of loop-2 might have a significant role in the interaction between meizothrombin and fibrinogen (Fig. 8B). The mutation of Asp554 (Asp222, chymotrypsin numbering), adjacent to the salt-bridging Arg253 (Arg231A, chymotrypsin numbering), could similarly be related to the conformational changes that are responsible for the differences between fibrinogen and protein C cleavage by the mutant meizothrombins. In conclusion, it is proposed that interactions within thrombin that involve autolytic loop-2 and the Na⁺-binding site primarily enhance thrombin action on fibrinogen, but impair thrombin action on protein C.

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