Recombinant human prothrombin (rII) and two mutant forms (R155A,R271A,R284A (rMZ) and R271A,R284A (rMZdesF1)) were expressed in mammalian cells. Following activation and purification, recombinant thrombin (rIIa) and stable analogues of meizothrombin (rMZa) and meizothrombin(desF1) (rMZdesF1a) were obtained. Studies of the activation of protein C in the presence of recombinant soluble thrombomodulin (TM) show TM-dependent stimulation of protein C activation by all three enzymes and, in the presence of phosphatidylserine/phosphatidylcholine phospholipid vesicles, rMZa is 6-fold more potent than rIIa. In the presence of TM, rMZa was also shown to be an effective activator of TAFI (thrombin-activatable fibrinolysis inhibitor) (Bajzar, L., Manuel, R., and Nesheim, M. E. (1995) J. Biol. Chem. 270, 14477–14484). All three enzymes were capable of inducing platelet aggregation, but 60-fold higher concentrations of rMZa and rMZdesF1a were required to achieve the effects obtained with rIIa. Second order rate constants (m−1 min−1) for inhibition by antithrombin III (AT-III) were 2.44 × 107 (rIIa), 6.10 × 106 (rMZa), and 1.05 × 102 (rMZdesF1a). The inhibition of rMZa and rMZdesF1a by AT-III is not affected by heparin. All three enzymes behave similarly to hirudin. The results of this and previous studies imply that full-length meizothrombin has marginal procoagulant properties compared to thrombin. However, meizothrombin has potent anticoagulant properties, expressed through TM-dependent activation of protein C, and can contribute to downregulation of fibrinolysis through the TM-dependent activation of TAFI.

Prothrombin is the inactive precursor of thrombin, a multifunctional serine protease that plays a central role in hemostasis. During the activation of prothrombin to thrombin by the prothrombinase complex, the active intermediate meizothrombin is generated initially (1–4). Meizothrombin is formed by the hydrolysis of Arg155-Thr156 by factor Xa (2, 5). Unlike thrombin, meizothrombin retains the two kringle domains and the γ-carboxyglutamyl acid (Gla)3 domain which is involved in the calcium-dependent interaction of prothrombin with phospholipid surfaces (6, 7). Cleavage of the peptide bond between Arg152-Ser156 in meizothrombin by thrombin-like activity generates another enzymatically active intermediate called meizothrombin(desF1) (8).

When the properties of meizothrombin and meizothrombin(desF1) are compared to those of thrombin, numerous similarities and differences emerge. Bovine meizothrombin and meizothrombin(desF1) have activities comparable to thrombin toward small substrates such as S-2238 (2, 9, 10). However, their activities toward macromolecular substrates such as fibrinogen or platelets are reduced (9, 10). As measured in Factor V-deficient plasma, bovine meizothrombin and meizothrombin(desF1), respectively, exhibit 1.4% and 12% of the activity of thrombin in the activation of Factor V (9). In the presence of negatively charged phospholipids, however, recombinant human meizothrombin (prothrombin R155A) is a potent Factor V activator (11). Although bovine meizothrombin (9) and meizothrombin(desF1) (12) can activate protein C (PC) in a thrombomodulin (TM)-dependent reaction, several studies have been unable to demonstrate significant binding between cell-surface recombinant TM and recombinant meizothrombin derived from the active site serine-to-alanine mutant human prothrombin (13, 14). Therefore, the conclusion was reached that meizothrombin is unlikely to be an important TM-dependent protein C activator (13). The inhibition of purified meizothrombin by antithrombin III (AT-III) has not been studied; however, meizothrombin(desF1) (15–17) is inhibited by antithrombin III with a second order rate constant approximately 2–3-fold lower than that for α-thrombin, and the interaction is not promoted by heparin (16, 17). Active-site blocked meizothrombin was also shown to have no heparin-neutralizing properties as opposed to active-site blocked thrombin (18).

Evidence obtained in vitro indicates that meizothrombin is a major intermediate during the clotting of whole blood (19). Similarly, a study ex vivo using human umbilical vein endothelial cells demonstrated the formation of meizothrombin and the

The abbreviations used are: Gla, γ-carboxyglutamyl acid; DAPA, dapsylarginine N-(3-ethyl-1,5-pentanediyl)amide; PCPS, phosphatidylcholine/phosphatidylserine (3:1) vesicles; S-2238, 3-phenylalanilayl-L-pipocetyl-L-arginyl-p-nitroaniline dihydrochloride; PPA-CCK, N-Pro-Arg chloromethylketone dihydrochloride; TM, thrombomodulin (Solulin, Ref. 28); rMZ, recombinant human prothrombin (R155A,R271A,R284A); rMZa, activated rMZ; rMZdesF1, recombinant human prothrombin (R271A,R284A); rMZdesF1a, activated rMZdesF1; TAFI, thrombin-activatable fibrinolysis inhibitor; AT, antithrombin; PAGE, polyacrylamide gel electrophoresis; APC, activated protein C.
Function of Meizothrombin and Meizothrombin(desF1)

accumulation of meizothrombin(desF1) during prothrombin activation (20). Meizothrombin was also shown to have vasoconstrictive activity 5-fold greater than that of α-thrombin (21).

Together, these studies suggest that meizothrombin and/or meizothrombin(desF1) produced in vivo have functions different from those of thrombin. Studies of these two intermediates in vitro, however, are complicated by their transient nature resulting in their conversion to thrombin. Previously, we reported the expression in mammalian cells of the recombinant human prothrombin derivative rMZ (R155A,R271A,R284A) (4), which can be activated to a stable form of meizothrombin (rMZa).

A second prothrombin derivative rMZdesF1 (R271A,R284A) (10) which can be activated to a stable form of meizothrombin(desF1) (rMZdesF1a) was also produced. The molecules were stabilized via arginine to alanine mutations at one of the two factor Xa cleavage sites and either one or both of the thrombin cleavage sites. The enzymatic activities of rMZa and rMZdesF1a toward fibrinogen and small substrates (4, 10) were similar to those described by others (2, 9) in studies of native meizothrombin. In addition, the Ca2+ and phospholipid binding properties of prothrombin are retained by rMZa (4). Thus, these activated mutants are useful surrogates for meizothrombin and meizothrombin(desF1). Consequently, the present studies were undertaken to analyze their functional properties and compare them to those of recombinant human thrombin (rHa).

**EXPERIMENTAL PROCEDURES**

**Materials**— Dulbecco’s modified Eagle’s medium, Ham’s F-12 (1:1), Opti-MEM, UltraTro G, and newborn calf serum were purchased from Life Technologies, Inc. Methotrexate was from David Bull Laboratories (Mulgrave, Victoria, Australia), and vitamin K1 was purchased from Sabex (Boucherville, Quebec) or Abbott Laboratories (Montreal, Quebec). The BHK cell line and pNUT vector were gifted from Dr. Richard Palmiter (Howard Hughes Medical Institute, University of Washington). The chromogenic substrates S-2238 and S-2666 were from Helena Laboratories (Mississauga, Ontario). Phospholipid vesicles (75% PC/25% PS (w/w)) were prepared as described previously (22). Human Factor X (1), Factor V (23), fibrinogen (24), and protein C (25) were purified and activated as described previously. TAFI (thrombin-activatable fibrinolysis inhibitor) was isolated by anion exchange, gel filtration, and plasminogen-Sepharose affinity chromatography as described previously (26). The purity of all proteins was verified by SDS-PAGE analysis. Additionally, thrombin activity was absent from the protein C and TAFI preparations as assayed by S-2238. Antithrombin III was characterized as described (31). For protein production, the cells were seeded in roller flasks and grown to a density of 10^7 cells/ml in F12 medium-F12 (1:1) supplemented with 5% newborn calf serum and 440 μM methotrexate. After 3–4 days, the cells were washed with Dulbecco’s modified Eagle’s medium-F12 and subsequently cultured in 0.5% UltraTro G or Opti-MEM (supplemented with 50 μg/mL zinc) containing 10 μg/mL vitamin K1. With the exception of the 1st day, the medium was collected every 150–200 ml, pooled, and stored at 4°C. The conditioned medium was concentrated using an Amicon CH2 centrifugor with a 0.1Y10 spiral cartridge, and rMZ was purified as described previously (4). rMZdesF1a was purified similarly except that the chromatography using a Ca2+ gradient was omitted.

**Activation of the Recombinant Proteins**—Wild type recombinant human prothrombin (rIIa) was activated by addition of the prothrombinase complex Factor Va, 10 μM phospholipid vesicles, and 5 mM CaCl2 directly to the conditioned medium (Opti-MEM). The activation mixture was then purified on an SP-50 column (2 ml) equilibrated with 20 mM HEPES, 150 mM NaCl, pH 7.4 at 22°C. Recombinant thrombin (rIIa) was eluted from the column with 20 mM HEPES, 0.5 mM NaCl, pH 7.4. Fractions containing rIIa were identified by amidolytic activity using S-2238. This procedure yielded pure rIIa as judged by SDS-PAGE, with 100% activity as measured by active-site titration. Both rMZ and rMZdesF1a were activated with ecarin. Briefly, ecarin (1.5 μg/ml) was added to the pure protein in 20 mM HEPES, 150 mM NaCl, 5 mM CaCl2 at 22°C. After 45 min, the activation mixture was chromatographed at 22°C on a column of benzamidine-Sepharose (27) equilibrated in 20 mM HEPES, 150 mM NaCl, 5 mM CaCl2, pH 7.4. Active fractions were then eluted with the remaining 10 mM benzamidine. Fractions containing the proteases were identified by the Bio-Rad protein assay. The pooled fractions containing the active proteins were dialyzed against 20 mM HEPES, 150 mM NaCl, 5 mM CaCl2, pH 7.4, and then concentrated, if necessary, using a Centricon PM30 membrane (Amicon, Danvers, MA). For rMZdesF1a, the removal of fragment 1 was complete with no zymogen remaining, as indicated by SDS-PAGE analysis under reducing conditions. This suggests that the feedback cleavage at position Arg535-Arg536 is carried out by meizothrombin/meizothrombin(desF1) and does not require the presence of endogenously formed α-thrombin. Both rMZa and rMZdesF1a were found to be 80–85% active.

**Active-site Titration and Displacement of DAPA by Hirudin**—The concentration of active protease rMZa, rMZdesF1a, and rMZdesF1b was determined by titration with PPA-CK. Small aliquots (3.2 μl) of PPA-CK (5 μM) were added to a protein sample (1.6 ml at 100 nm) containing 200 nM DAPA, and the decrease in fluorescence due to the displacement of DAPA from the active site was monitored (λex = 280 nm, λem = 545 nm) in a Perkin-Elmer LS-50B fluorescence spectrophotometer. Plots of fluorescence intensity versus the concentration of PPA-CK were constructed, and extrapolated to the baseline fluorescence values yielded the active-site concentrations of each protease. The interaction of the recombinant proteins with hirudin was measured similarly. Briefly, small aliquots (3.2 μl) of hirudin (5 μM) were added to a protein sample (1.6 ml at 100 nm) containing 200 nM DAPA, and the decrease in fluorescence was monitored (λex = 280 nm, λem = 545 nm). The displacement of DAPA by hirudin was considered to be the result of two competing equilibrium binding interactions, E-b = E-D, characterized by dissociation constants Kep and Kep (E, H, and D represent enzyme, hirudin, and DAPA, respectively). By utilizing the binding equations [E][H] = Kep[EH] and [E][D] = Kep(E-D), plus the conservation equations [E] = [E] + [E-D] + [EH], and [H][D] = [H] + [EH], the following equation was derived, which relates fluorescence intensity (proportional to [E-D]) with proportionality constant C; the concentrations of hirudin, enzyme, and DAPA and the dissociation constants for the enzyme-DAPA and the enzyme-hirudin interactions.

**Platelet Aggregation**—Platelets were prepared from freshly drawn blood using a modification of the method clamped et al. (31). Platelets were washed once in TA buffer (137 mM NaCl, 2.7 mM KCl, 12 mM NaHCO3, 0.42 mM Na2HPO4, 1.0 mM MgCl2, 5.6 mM glucose, 2.5 mg/ml bovine serum albumin, pH 7.35) containing 30 μg/ml apyrase, 5 μM prostaglandin E1, 28 units/ml hirudin, and washed again in the same buffer without hirudin. The platelets were finally resuspended in TA buffer containing 50 mM HEPES, 3 μg/ml apyrase (HTA). The number of platelets in a small aliquot (10 μl) was deter-
human protein C was incubated with various combinations of recombinant proteins (rIIa, rMZa, or rMZdesF1a) (5.8 nM final) was incubated with 20 mM HEPES, 150 mM NaCl, 5 mM CaCl$_2$, pH 7.4, for 10 min at 37°C in the presence of thrombomodulin. Rates of protein C activation were calculated from the concentration of TM.

Results and Discussion


ting the substrate. The protein C activation was determined from the rates of change of the substrate. The protein C activation was measured after 10 min of incubation. The data were subsequently fit by non-linear regression analysis to the saturation equation $\Delta [\text{APC}] = \frac{V_{max}}{K_m + [\text{PCPS}]}$ where $V_{max}$ is the maximum increment in rate achieved with PCPS, $K_m$ is the rate at the initial concentration. The analysis of the data indicated saturation in the presence of thrombomodulin.


tation—Inhibition by Antithrombin III—For each assay, 100 pM of enzyme (rIIa, rMZa, or rMZdesF1a) (5.8 nM final) was incubated with 20 μM of AT-III (final concentrations 0 to 3.87 μM) at 22°C for 0 to 30 min. The remaining activity was then assayed by adding 10 μM of the sample to 190 μM of S-2238 (final concentration 0.4 mM in 20 mM HEPES, 150 mM NaCl, 5 mM CaCl$_2$, 0.01% Tween 80, pH 7.4). The conversion of S-2238 was followed by monitoring the absorbance at 405 nm at 30-s intervals for up to 30 min at 37°C in a Titre-Tek Twin reader. The data were plotted as ln[active enzyme] versus time, and, from the slopes, the pseudo-first order rate constants were calculated. The second order rate constants were calculated from plots of the slopes of the pseudo-first order rate constants versus the AT-III concentrations. For the inhibition by AT-III in the presence of heparin, a protein sample (rIIa, rMZa, or rMZdesF1a) (1.6 ml, 25 nM) in 20 mM HEPES, 150 mM NaCl, 5 mM CaCl$_2$, 0.01% Tween 80, pH 7.4) was added at 22°C in a Perkin-Elmer MPF-66 fluorescence spectrophotometer. A small aliquot of AT-III (1.0 μM final concentration) was added, and fluorescence was monitored (λ$_{ex}$ = 280 nm, λ$_{em}$ = 545 nm). At 100 nM heparin (6 units/ml) was added to the sample, and the displacement of DAPA from the active site by AT-III over time was monitored by fluorescence.


tinued in a Baker System 9000 Cell Counter. Aggregation studies were carried out in a single-channel Payton Aggregation Module (Payton Scientific, Buffalo, NY) using siliconized glass cuvettes. Gain settings were adjusted to give 90% transmittance for HTA buffer and 10% for the starting platelet suspension. Varying amounts of rIIa, rMZa, and rMZdesF1a were added to platelets (1 × 10$^8$/ml) in HTA buffer containing 2 mM CaCl$_2$. The aggregation of the platelets was followed continuously by measuring the percentage of the increment in light transmittance with time. The platelet preparation was tested for its response to rIIa (0.8 μM) at the beginning and the end of the experiment.


trate of protein C activation. The aggregation of the platelets was followed continuously by measuring the percentage of the increment in light transmittance with time.


t. The aggregation of the platelets was followed continuously by measuring the percentage of the increment in light transmittance with time.


tation by Ad-APC—Termination of protein C activation by activated TAFI (TAFIa) was monitored at 254 nm in a microtiter plate. The reactions were initiated by the addition of 150 mM CaCl$_2$. The aggregation of the platelets was followed continuously by measuring the percentage of the increment in light transmittance with time.


tation by Ad-APC—Termination of protein C activation by activated TAFI (TAFIa) was monitored at 254 nm in a microtiter plate. The reactions were initiated by the addition of 150 mM CaCl$_2$. The aggregation of the platelets was followed continuously by measuring the percentage of the increment in light transmittance with time.


tation by Ad-APC—Termination of protein C activation by activated TAFI (TAFIa) was monitored at 254 nm in a microtiter plate. The reactions were initiated by the addition of 150 mM CaCl$_2$. The aggregation of the platelets was followed continuously by measuring the percentage of the increment in light transmittance with time.


tation by Ad-APC—Termination of protein C activation by activated TAFI (TAFIa) was monitored at 254 nm in a microtiter plate. The reactions were initiated by the addition of 150 mM CaCl$_2$. The aggregation of the platelets was followed continuously by measuring the percentage of the increment in light transmittance with time.


tation by Ad-APC—Termination of protein C activation by activated TAFI (TAFIa) was monitored at 254 nm in a microtiter plate. The reactions were initiated by the addition of 150 mM CaCl$_2$. The aggregation of the platelets was followed continuously by measuring the percentage of the increment in light transmittance with time.


tation by Ad-APC—Termination of protein C activation by activated TAFI (TAFIa) was monitored at 254 nm in a microtiter plate. The reactions were initiated by the addition of 150 mM CaCl$_2$. The aggregation of the platelets was followed continuously by measuring the percentage of the increment in light transmittance with time.


tation by Ad-APC—Termination of protein C activation by activated TAFI (TAFIa) was monitored at 254 nm in a microtiter plate. The reactions were initiated by the addition of 150 mM CaCl$_2$. The aggregation of the platelets was followed continuously by measuring the percentage of the increment in light transmittance with time.
in Fig. 2 and Table II. Phospholipid vesicles had no effect on protein C activation by the rIIa-TM complex. In contrast, upon addition of PCPS vesicles, the rate approximately doubled with rMZdesF1a-TM and increased by approximately 13-fold with rMZA-TM. At saturating levels of PCPS, rMZA-TM thus catalyzes protein C activation at a rate which is ~6-fold greater than that for rIIa-TM. These data demonstrate not only that rMZA and rMZdesF1a can activate protein C in the presence of TM, but also that the rate achieved in the presence of phospholipid vesicles and rMZA appreciably exceeds that observed with rIIa.

A second substrate was recently identified for the thrombin-TM complex: TAFI (32). We therefore investigated the ability of rMZA to activate TAFI. The data displayed in Fig. 3 indicate the effect of TM on the rMZA-catalyzed proteolytic activation of TAFI. The experiments were performed at a range of initial concentrations of TAFI (0.26 to 1.75 μM). As the data indicate, saturation of rates was observed with respect to the TAFI concentration at all TM concentrations. The rates also showed saturation with respect to the TM concentration at all of the TAFI concentrations. The *Kₘ* values for TAFI did not vary with TM concentrations, but *Vₘₐₓ* values showed saturation. All of the data therefore were fit to the single equation indicated under “Experimental Procedures.” The lines in Fig. 3 were obtained from regression analyses of the data. The fit was such that the residuals were distributed randomly and no individual datum deviated by more than 15% of the value predicted from the rate equation, thus indicating that the equation and implied model represent the data well. The values of parameters that were returned by the non-linear regression analysis were: *Kₘ = 0.7 μM*, *k₉ = 0.08 s⁻¹*, and *Kₐ = 2.6 nM* (for the rMZA-TM interaction). The *Kₐ* value inferred from the kinetics of TAFI activation is lower than that for protein C activation. This may be due to the difference in temperature (37 °C for protein C versus 22 °C for TAFI) or may reflect differential contributions of the two proteins (TAFI and protein C) in the formation of the ternary complex. The model which fits these data (3) implies a mechanism whereby rMZA can interact with either TM or TAFI to form the respective binary complexes, and then the third component can be added to form a ternary rMZA-TM-TAFI complex, in which activation of TAFI occurs. The primary effect of TM is to increase the *k₉*. This shows that rMZA is a potent activator of TAFI when complexed to TM.

**TABLE I**

| Function of Meizothrombin and Meizothrombin(desF1) |
|-----------------|-----------------|-----------------|
| Enzyme          | [Enzyme] | *Kₘ* | *k₉* |
|-----------------|-------|------|------|
| rIIa            | 2.0   | 24 ± 1 | 4.7 ± 0.1 |
| rMZA            | 0.5   | 72 ± 3 | 5.8 ± 0.1 |
| rMZdesF1a       | 2.0   | 63 ± 4 | 4.7 ± 0.2 |
| rMZdesF1a       | 0.5   | 33 ± 2 | 4.3 ± 0.1 |
| rMZdesF1a       | 2.0   | 35 ± 3 | 4.0 ± 0.1 |

Thrombin is a potent physiological activator of platelet aggregation. We tested whether the three recombinant enzymes were capable of inducing platelet aggregation. rMZA and rMZdesF1a were both approximately 60-fold less effective than rIIa with respect to the concentration required to give maximal rates of aggregation. As shown in Fig. 7, 0.8 nM rIIa induced a maximal rate of platelet aggregation (22% T/min), and approximately 50 nM concentrations of either rMZA or rMZdesF1a were lower (6.10 × 10⁴ and 1.05 × 10⁵ M⁻¹ min⁻¹, respectively). rMZA (which retains the Gla domain) was twice as resistant as rMZdesF1a to inhibition by AT-III, suggesting that the fragment I domain may further reduce the interaction between the two molecules. The inhibition of the three recombinant enzymes also was monitored by measuring the decrease in fluorescence due to the displacement of the active-site fluorescent probe DAPA by AT-III (Fig. 5). Under these conditions, upon the addition of heparin, a rapid decrease in the concentration of the rIIa-DAPA complex was observed, while the concentrations of the DAPA complexes with rMZA and rMZdesF1a were unchanged. These data confirm reports by others that inhibition of thrombin by AT-III is promoted by heparin, whereas the inhibition of meizothrombin (2, 9) or meizothrombin(desF1) (16, 17) is not.

Hirudin is a highly specific thrombin inhibitor currently in clinical trials for the prevention of thrombosis. We investigated whether rMZA and rMZdesF1a would be sensitive to hirudin inhibition. The interactions of hirudin with the three recombinant enzymes was analyzed by measuring the decrement in fluorescence as hirudin displaces DAPA from the active site (Fig. 6). rIIa, rMZA, and rMZdesF1a were similarly affected by the leech inhibitor. Although the rMZA-DAPA complex exhibits greater fluorescence intensity than rIIa-DAPA or rMZdesF1a-DAPA, all three enzymes yielded baseline fluorescence at the same hirudin concentration. The dissociation constants (φ₁₃) determined by non-linear regression analyses for the interactions of hirudin with the three enzymes were: 134 ± 36 (rIIa), 355 ± 62 (rMZA), and 231 ± 37 (rMZdesF1a). These results suggest that the hirudin binding sites are similarly or possibly equally accessible in meizothrombin, meizothrombin(desF1), and thrombin.

Fig. 2. Phospholipid dependence of protein C activation. The enzymes rIIa (○), rMZdesF1a (●), and rMZA (●) were incubated in 5 mM CaCl₂ with protein C (1.0 μM), soluble TM (40 nM), and PCPS vesicles at the concentrations indicated on the horizontal axis, for 10 min at 37 °C. Concentrations of APC were then determined from the rates of hydrolysis of the substrate S-2366. Rates are expressed relative to the rates observed without PCPS, which were 3.54 (○), 2.25 (●), and 1.70 (●) in units of mol of APC/min/mol of enzyme. The data were analyzed by non-linear regression analyses, the results of which are tabulated in Table II.
Effect of PCPS vesicles on the thrombomodulin-enhanced activation of protein C by rIIa, rMZa, and rMZdesF1a

Data were fitted by non-linear regression to the equation \( R = R_0 + \Delta R_{\text{max}}/([\text{PCPS}]/K_d + [\text{PCPS}]) \) where \( R_0 \) is rate in the absence of PCPS and \( \Delta R_{\text{max}} \) is the maximum increment obtained with PCPS. \( K_d \) is the PCPS concentration required to achieve half of the maximal rate divided by the rate in the absence of PCPS \((R_0 + \Delta R_{\text{max}})/R_0\). The concentrations of TM and PC were 40 nM and 1.0 μM, respectively.

| Enzyme          | \( R_0 \) | \( R_{\text{max}} \) | \( \Delta R_{\text{max}} \) | \( K_d \) | -Fold increase |
|-----------------|-----------|----------------------|-----------------------------|----------|---------------|
|                 | min \(^{-1}\) | min \(^{-1}\) | min \(^{-1}\) | μM       |               |
| rIIa            | 3.54 ± 0.02 | 3.54 ± 0.02 | NA \(^a\) | NA       | NA            |
| rMZa            | 1.70 ± 0.18 | 22.20 ± 0.70 | 20.50 ± 0.70 | 141 ± 14 | 13.1\(^b\)   |
| rMZdesF1a       | 2.25 ± 0.04 | 4.77 ± 0.20 | 2.52 ± 0.20 | 185 ± 38 | 2.1          |

\(^a\) NA, not applicable.

\(^b\) The 13.1-fold increase refers to the rate of the \( R_{\text{max}} \) to \( R_0 \). The 6-fold increase of the rate of rMZa to that of rIIa saturating PCPS is given by the ratio of the respective \( R_{\text{max}} \) values.

**DISCUSSION**

In this study, we investigated the activities of rMZa and rMZdesF1a to determine their relative effects on procoagulant and anticoagulant reactions. Table III summarizes these properties relative to rIIa. Both rMZa and rMZdesF1a show poor procoagulant function as demonstrated by their fibrinogen clotting and platelet aggregation activities. Although both enzymes were capable of initiating platelet aggregation, peak rates of aggregation required 60-fold higher concentrations of rMZa or rMZdesF1a compared to rIIa. In addition, these peak rates were lower than that of rIIa. These results suggest that rMZa and rMZdesF1a interact less favorably with the thrombin receptor described by Vu et al. (33) and/or act through a different receptor.

In contrast, both rMZa and rMZdesF1a exhibit a TM-dependent maximum rate of protein C activation that is similar to that of rIIa, in the absence of phospholipids. In the presence of phospholipid vesicles, the maximum rate for rMZa is 6-fold higher than that of rIIa. The TM-dependent activation of TAFI was catalyzed at a significant rate by rMZa, but still only 10% that of rIIa-TM (32). Both rMZa and rMZdesF1a were inhibited by AT-III although 2- to 4-fold less efficiently than rIIa.

Meizothrombin and thrombin display both procoagulant and anticoagulant activities. When rMZa is compared to rIIa, however, its anticoagulant properties predominate. One possible exception to this interpretation comes from the data reported by Tans et al. (11) which show that a recombinant form of human meizothrombin (prothrombin R155A) activates factor V at 4 times the rate of thrombin in the presence of 90% PC/10% PS phospholipid vesicles, but at a much lower rate (70-fold) in their absence. Furthermore, our data on protein C and TAFI activation indicate TM-dependent potentiation of rMZa activity. These observations appear inconsistent with the report by Wu et al. (13) who were unable to demonstrate significant binding between recombinant active-site serine-to-alanine mutant meizothrombin and cell surface recombinant TM. Differences in the TM species may account for some of the discrepancies between our results and those of others. Our study employs a recombinant soluble form of thrombomodulin containing four mutations and lacking chondroitin sulfate, whereas Wu et al. (13) utilized cellular thrombomodulin. Thus, results with soluble thrombomodulin and synthetic vesicles probably cannot be reliably extrapolated to infer characteristics of protein C activation on cellular surfaces by meizothrombin. Further studies with cell surface or membrane-bound thrombomodulin would be needed to rationalize the apparent discrepancy. A possible source of error in our experiment could be a contamination of both protein C and TAFI. We feel that this is not the case because the control experiments without enzyme showed no activation of protein C or TAFI or endogenous amidolytic activity. In addition, the potentiation of protein C activation by phospholipids with rMZa, but not rIIa, clearly indicates that catalysis is due to rMZa rather than contaminating thrombin.

Our data provide some insight into the functionality of the fragment 1 domain and the two potential anion binding exosites of meizothrombin. The marked stimulation of protein C activation by PCPS vesicles with rMZa, but not rMZdesF1a or rIIa, clearly indicates the importance of the Gla domain for potentiation of the reaction by phospholipids. Furthermore, assuming that rMZa, rMZdesF1a, and rIIa interact similarly with TM, our results imply that exosite 1 is available on rMZa and rMZdesF1a. This is consistent with the work by Ni et al. (34) who demonstrated by NMR spectroscopy the partial accessibility of exosite 1 on prothrombin, prethrombin-1, and meizothrombin(desF1).

The reduced second order rate constants for inhibition of rMZa and rMZdesF1a by AT-III, compared to that of rIIa, confirms previous observations (15) that the fragment 2 domain, either covalently attached to the protease domain or non-covalently associated with it, attenuates the interaction.
between AT-III and the protease domain. As reported previously (2, 16, 17), the inhibition of meizothrombin (desF1) is not promoted by heparin. The present work shows that this is also true for rMZa. These observations imply that the covalently associated fragment 2 domain blocks in the protease domain the anion binding exosite 2 which has been shown in thrombin to interact with heparin (35–37). Thus, the fragment 2 domain of rMZa and rMZ(desF1a) prevents formation of the ternary AT-III-protease-heparin complex required for stimulated inhibition by AT-III (38). The reduced rate constant for inhibition of rMZa by AT-III, compared to that of thrombin, suggests that rMZa would have a longer half-life in circulation than thrombin, and inhibition of rMZa and rMZ(desF1a) would not be influenced by heparin. In contrast, rMZA, rMZ(desF1a), and rIIa would likely be inhibited similarly by hirudin in vivo.

Our studies, along with those of others, show that both rMZA (and presumably native meizothrombin) and thrombin possess procoagulant, anticoagulant, and antifibrinolytic activities. The latter two activities are expressed through the TM-dependent activation of protein C and TAFI, respectively. Thrombin and meizothrombin clearly are not equipotent in these three activities. Meizothrombin is only 10% or less effective as thrombin in expressing procoagulant and antifibrinolytic activities, as defined by fibrinogen clotting, platelet activation, and TAFI activation. However, it expresses substantial anticoagulant activity, as defined by protein C activation, which approaches that of thrombin in the absence of phospholipids and exceeds it severalfold in the presence of phospholipids. These properties of meizothrombin suggest that it plays a significant role in the control of hemostasis, perhaps different from that of thrombin. This would be especially so in small vessels which are dense with TM. Thus, because prothrombin activation generates two enzymes, each with unique properties, two distinct states are conceivably possible. One state, extant when meizothrombin predominates, would be relatively anticoagulant and profibrinolytic. This state also would likely be self-limiting because of negative feedback through the anticoagulant pathway. The other state, extant when thrombin predominates, would be relatively procoagulant and antifibrinolytic. If mechanisms ex-

![Figure 4](image1.png)

**FIG. 4.** Inhibition by antithrombin-III. The proteases were incubated with AT-III (1.29 μM in this instance) at 22°C for 0 to 30 min, and the remaining activity was measured at the indicated times by assays with S-2238 for rIIa (○), rMZ(desF1a) (▲), and rMZA (●). Pseudo-first order rate constants are plotted versus the AT-III concentration in the inset. These plots yielded second order rate constants (s⁻¹) of 2.44 × 10⁵ (rIIa), 1.05 × 10⁶ (rMZ(desF1a)), and 6.10 × 10⁴ (rMZA).

![Figure 5](image2.png)

**FIG. 5.** Inhibition by AT-III in the presence of heparin. rIIa (○), rMZ(desF1a) (▲), and rMZA (●) were incubated with AT-III (1.0 μM) in the presence of DAPA (500 nM) for 100 s, at which time heparin (6 units/ml) was added. The fluorescence intensity was monitored at 545 nm with an excitation wavelength of 280 nm. The results are expressed as a fraction of DAPA-bound enzyme remaining.

![Figure 6](image3.png)

**FIG. 6.** Displacement of active-site-bound DAPA by hirudin. The displacement of fluorescent probe DAPA from the active site by hirudin was monitored at 545 nm with an excitation wavelength of 280 nm for rIIa (○), rMZ(desF1a) (▲), and rMZA (●). Data were analyzed by non-linear regression, which yielded Kₐ values for the enzyme-hirudin interactions of 134 pM (rIIa), 355 pM (rMZA), and 231 pM (rMZ(desF1a)).
and the other as procoagulant and antifibrinolytic. Could be characterized as anticoagulant and profibrinolytic, then prothrombin activation could potentially lead to, or be switched toward, two distinct end points, one of which could be characterized as anticoagulant and profibrinolytic, and the other as procoagulant and antifibrinolytic.

**FIG. 7.** Platelet aggregation. The percentage of light transmittance as a function of time was monitored to measure the rate of platelet aggregation by rIIa (○), rMZdesF1a (▲), and rMZa (●).

**TABLE III**

Relative activities of rIIa, rMZa, and rMZdesF1a toward physiological substrates and inhibitors

| Activity                    | Enzyme | rIIa | rMZa | rMZdesF1a |
|-----------------------------|--------|------|------|-----------|
| Fibrinogen clotting         |        | 1.00 | 0.07 | 0.27      |
| Platelet aggregation        |        | 1.00 | 0.02 | 0.02      |
| Protein C + TMf              |        | 1.00 | 0.83 | 0.73      |
| Protein C + TM + PCPS        |        | 1.00 | 6.27 | 1.35      |
| TAFI + TMf                   |        | 1.00 | 0.10 | NDf       |
| AT-IIIg                      |        | 1.00 | 0.43 | 0.25      |

- a From Stevens et al. (10).
- b From concentration dependence, Fig. 7.
- c From average $R_{\text{max}}$ values, Table I.
- d From $k_{\text{max}}$ values, Table II.
- e From $k_{\text{max}}$ values for TAFI activation by rMZa, Fig. 3, and plasma-derived thrombin (Bazjar et al. (32)).
- f ND, not determined.
- g From second order rate constants, Fig. 4.

ist in vivo which favor either meizothrombin or thrombin generation, then prothrombin activation could potentially lead to, or be switched toward, two distinct end points, one of which could be characterized as anticoagulant and profibrinolytic, and the other as procoagulant and antifibrinolytic.

**REFERENCES**
1. Krishnaswamy, S., Mann, K. G., and Nesheim, M. E. (1986) *J. Biol. Chem.* 261, 8977–8984.
2. Rosing, J., Zwaal, R. F. A., and Tans, G. (1986) *J. Biol. Chem.* 261, 4224–4228.
3. Boskovic, D. S., Giles, A. R., and Nesheim, M. E. (1990) *J. Biol. Chem.* 265, 10497–10505.
4. Côté, H. C. F., Stevens, W. K., Bazjar, L., Banfield, D. K., Nesheim, M. E., and MacGillivray, R. T. A. (1994) *J. Biol. Chem.* 269, 11374–11380.
5. Owen, W. G., Esmon, C. T., and Jackson, C. M. (1974) *J. Biol. Chem.* 249, 594–605.
6. Nelsestuen, G. L. (1976) *J. Biol. Chem.* 251, 5648–5656.
7. Nelsestuen, G. L., Broderius, M., and Martin, G. (1976) *J. Biol. Chem.* 251, 6898–6899.
8. Morita, T., Iwanaga, S., and Suzuki, T. (1976) *J. Biochem. (Tokyo)* 79, 1089–1108.
9. Doyle, M. F., and Mann, K. G. (1990) *J. Biol. Chem.* 265, 10693–10701.
10. Stevens, W. K., Côté, H. C. F., MacGillivray, R. T. A., and Nesheim, M. E. (1996) *J. Biol. Chem.* 271, 8062–8067.
11. Tans, G., Nicolaas, G. A. F., Thomassen, M. C. L. G. D., Hemker, H. C., van Zonneveld, A. J., Vannekloek, H., and Rosing, J. (1994) *J. Biol. Chem.* 269, 15969–15972.
12. Liu, L.-W., Rezaie, A. R., Carson, C. W., Esmon, N. L., and Esmon, C. T. (1994) *J. Biol. Chem.* 269, 11807–11812.
13. Wu, Q., Tsai, M., Lenz, S. R., and Sadler, J. E. (1992) *J. Biol. Chem.* 267, 7083–7088.
14. Wu, Q., Picard, V., Aiach, M., and Sadler, J. E. (1994) *J. Biol. Chem.* 269, 3725–3730.
15. Walker, F. J., and Esmon, C. T. (1979) *J. Biol. Chem.* 254, 5618–5622.
16. Schoen, P., and Lindhout, T. (1987) *J. Biol. Chem.* 262, 11268–11274.
17. Lindhout, T., Baruch, D., Schoen, P., Franssen, J., and Hemker, H. C. (1986) *Biochemistry* 25, 5962–5969.
18. Pieters, J., Franssen, J., Visch, C., and Lindhout, T. (1987) *Thromb. Res.* 45, 573–580.
19. Bovill, E. G., Tracy, R. P., Hayes, T. E., Jenny, R. J., Bhushan, F. H., and Mann, K. G. (1995) *Arterioscler. Thromb. Vasc. Biol.* 15, 754–758.
20. Tijburg, P. N. M., van Heerde, W. L., Leenhouts, H. M., Hessing, M., Bouma, B. N., and de Groot, P. G. (1991) *J. Biol. Chem.* 266, 4017–4022.
21. Thompson, L. P., Doyle, M. F., Mann, K. G., and Bevan, J. A. (1987) *Blood* 70, 410a (abstr. 1494).
22. Bloom, J. W., Nesheim, M. E., and Mann, K. G. (1979) *Biochemistry* 18, 4419–4425.
23. Nesheim, M. E., Katzman, J. A., Tracy, P. B., and Mann, K. G. (1981) *Methods Enzymol.* 80, 249–274.
24. Bazjar, L., and Nesheim, M. E. (1993) *J. Biol. Chem.* 268, 8608–8616.
25. Bazjar, L., Fredenburgh, J. C., and Nesheim, M. (1990) *J. Biol. Chem.* 265, 16948–16954.
26. Bazjar, L., Manuel, R., and Nesheim, M. E. (1995) *J. Biol. Chem.* 270, 14477–14484.
27. Nesheim, M. E. (1983) *J. Biol. Chem.* 258, 14708–14718.
28. Glaser, C. B., Morser, J., Clarke, J. H., Blasko, E., McLean, K., Kuhn, I., Lin, J.-H., Vilander, L., Andrews, W. H., and Light, D. R. (1992) *J. Clin. Invest.* 90, 2565–2573.
29. Lin, J.-H., McLean, K., Morser, J., Young, T. A., Wydro, R. M., Andrews, W. H., and Light, D. R. (1994) *J. Biol. Chem.* 269, 25021–25030.
30. Nesheim, M. E., Prendergast, F. G., and Mann, K. G. (1979) *Biochemistry* 18, 996–1003.
31. Mustard, J. F., Perry, D. W., Ardlie, N. G., and Packham, M. A. (1972) *Br. J. Haematol.* 22, 193–204.
32. Bazjar, L., Morser, J., and Nesheim, M. E. (1996) *J. Biol. Chem.* 271, 16663–16668.
33. Vu, T.-K., Hung, T. D., Wheaton, V. L., and Coughlin, S. R. (1991) *Cell* 64, 1057–1068.
34. Ni, F., Ning, Q., Jackson, C. M., and Fenton, J. W. II (1993) *J. Biol. Chem.* 268, 16899–16902.
35. Church, F. C., Pratt, C. W., Noyes, C. M., Kalayananit, T., Sherrill, G. B., Tobin, R. B., and Meade, J. B. (1989) *J. Biol. Chem.* 264, 18419–18425.
36. Gaz, Z.-R., Li, Y., Chen, Z., Lewis, S. D., and Shafar, J. A. (1994) *J. Biol. Chem.* 269, 1301–1305.
37. Sheehan, J. P., and Sadler, J. E. (1994) *Proc. Natl. Acad. Sci. U.S.A.* 91, 5518–5522.
38. Olson, S. T., Bjork, I., Sheffer, R., Craig, P. A., Shore, J. D., and Choay, J. (1992) *J. Biol. Chem.* 267, 12528–12538.