Calcium Ion Modulation of Meizothrombin Autolysis at Arg$^{55}$-Asp$^{56}$ and Catalytic Activity

(Received for publication, December 14, 1995)

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When a recombinant variant of prothrombin with the cleavage site mutations R155A, R271A, and R284A (rMZa) is exposed to either prothrombinase or ecarin, a form of meizothrombin (rMZa*) is generated that is stable for weeks in the presence of Ca$^{2+}$ (Côté, H. C. F., Stevens, W. K., Bajzar, L., Banfield, D. K., Nesheim, M. E., and MacGillivray, R. T. A. (1994) J. Biol. Chem. 269, 11374–11380). In the absence of Ca$^{2+}$, however, rMZa is rapidly cleaved within a disulfide bonded loop in the F1 domain at Arg$^{55}$ in the sequence RTPR, DKL, yielding a molecule with 3 chains joined by two disulfide bonds (rMZdesF1a). Cleavage kinetics are first order regardless of the rMZa concentration, indicating an intramolecular cleavage. This cleavage does not occur at Ca$^{2+}$ concentrations in excess of 1.0 mM. To assess the role of the F1 domain in rMZa activity, another variant lacking the R155A mutation (rMZdesF1) was expressed, which when activated yields meizothrombin lacking the F1 domain (rMZdesF1a). Rates of hydrolysis of the tripeptide substrate S2238 by rMZa or rMZa* increase from 60% to 90% that of recombinant thrombin as Ca$^{2+}$, Mg$^{2+}$, or Mn$^{2+}$ concentrations are varied from 0 to 10 mM. $K_m$ and $k_{cat}$ values for rMZa in the absence and presence of 5 mM Ca$^{2+}$ are 1.9 and 2.2 μM and 65 and 105 s$^{-1}$. TAME esterase activity of rMZa also increases with 5 mM Ca$^{2+}$. No such metal ion-dependent effects are obtained with either thrombin or rMZdesF1a. Fibrinogen clotting activities, relative to that of thrombin, increase in a manner analogous to those obtained with small substrates, for rMZa and rMZa* but not rMZdesF1a. Complexes of the active site probe dansylarginine N-(3-ethyl-1,5-pentanediyl)amide with rMZa and rMZa*, but not thrombin or rMZdesF1a exhibit large cation-dependent decreases in fluorescence intensity, suggesting that metal ion binding in the F1 domain alters the environment of the probe at the active site. These results indicate that in the absence of divalent cations, the activity of rMZa is inhibited, perhaps by obstruction of the active site by the F1 domain, and that Ca$^{2+}$ binding to the F1 domain modulates the properties of not only the F1 domain but the protease domain.

Prothrombin is one of a group of plasma proteins involved in blood coagulation that require vitamin K-dependent carboxy-

* This work was supported in part by Grants MA-9781 (to M. E. N.) and MT-7716 (to R. T. A. M.), by an Ontario Graduate Scholarship (to W. K. S.), and a Medical Research Council Studentship (to H. C. F. C.).

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‡ The abbreviations used are: Gla, γ-carboxyglutamic acid; DAPA, dansylarginine-N-(3-ethyl-1,5-pentanediyl)amide; BHK, baby hamster kidney cells; S2238, p-phenylalanyl-γ-pipeeryl-γ-arginyll-p-nitroaniline-dihydrochloride; TAME, p-tosyl-γ-arginine methyl ester.
activities at varying levels of Ca$^{2+}$.

To understand the consequences of retaining the amino-terminal portion of the prothrombin molecule on meizothrombin activity, recombinant prothrombin and two site-directed mutants were prepared. One mutant was prothrombin (R155A, R271A, R284A) designated rMZ, which upon activation with either the protrombinase complex or ecarin yields full-length meizothrombin (6). A second mutant prothrombin (R271A, R284A) designated rMZdesF1 yields meizothrombin des-fragment 1 upon activation. These and recombinant prothrombin (rI) were expressed, isolated, and activated, and their activities against small substrates and fibrinogen were compared in the presence of varying amounts of Ca$^{2+}$.

**EXPERIMENTAL PROCEDURES**

Expression Vectors—For expression in mammalian cells, the cDNAs for rI and rMZ were ligated into the pNUT expression vector (11) downstream of the zinc-inducible mouse metallothionein promoter and upstream of the human growth hormone polyadenylation signal, as described previously for rMZ (6). The expression vector for rMZdesF1 was prepared by digesting the pNUT vector with the restriction enzyme BstEII, liberating a 1-kilobase fragment corresponding to a small portion of the vector backbone and the first 690 base pairs of the rMZ cDNA. This fragment, containing the R155A mutation, was then replaced with the analogous fragment obtained from pNUT containing the wild-type prothrombin cDNA, yielding a construct coding for a mutant prothrombin (R271A, R284A), with the thrombin cleavage site Arg$^{55}$ in the absence of calcium ion to form a three-chain form of meizothrombin (rMZa).

Proteins—Human plasma factor V (12), factor X (13), and fibrinogen (13) were isolated, and factors V and X were activated as described previously. The prothrombin activator of Echis carinatus venom, ecarin, was purified from crude venom (Sigma) by anion exchange chromatography and polyacrylamide preparative gel electrophoresis as described previously (14). The recombinant proteins rMZ and rMZdesF1 were expressed and isolated using the methods described for rMZ by Côté et al. (6). Conditioned medium (Opti-Mem Life Technologies Inc.) supplemented with 50 μM ZnCl$_2$ was collected from stably transfected lines of BHK cells cultured at 37 °C in roller bottles. Sodium citrate was added to a final concentration of 0.025 M, and the prothrombin was adsorbed with the addition of 1 M BaCl$_2$ (80 ml/liter) and subsequent formation of a barium citrate precipitate. The barium citrate pellet was eluted with 0.2 M EDTA, and the eluate was concentrated and dialyzed against 0.02 M Tris, 0.15 M NaCl, pH 7.4. Subsequent ion exchange using a salt gradient and pseudo-affinity chromatography with a calcium ion gradient on a Pharmacia Mono Q column yielded homogenous species (6). Samples of the recombinant proteins rMZ and rMZdesF1 (1 mg at 0.1 mg/ml) were activated by incubation with ecarin (1.5 μg/ml) at 22 °C in the presence of 5 mM CaCl$_2$ for approximately 30 min. The activation mixtures were passed at 22 °C over a 2-ml column of benzo- midine Sepharose, previously equilibrated with 0.02 M HEPES, 0.15 M NaCl, 5 mM CaCl$_2$, pH 7.4. The column was washed with 20 ml of the equilibration buffer, and the enzymes were eluted with the same buffer supplemented with 10 mM benzamidine. Fractions containing rMZa or rMZdesF1a were identified using a Bio-Rad protein assay, mixing 90 μl of each fraction with 20 μl of the dye reagent, and monitoring color development at 595 nm. Fractions containing protein were pooled and dialyzed against 0.02 M HEPES, 0.15 M NaCl, 5 mM CaCl$_2$, pH 7.4, and subsequently stored at 4 °C. Recombinant thrombin was isolated by activating rI1 directly in the conditioned medium. Conditioned medium (40 ml of Opti-Mem) was treated with 5 mM factor Va, 5 mM factor X and 10 μM phosphatidylcholine/phosphatidylserine (3:1) vesicles and 5 mM CaCl$_2$. The activation mixture was passed over a 2 ml column of SP-C50 cation exchange resin, previously equilibrated with 0.02 M HEPES, 0.15 M NaCl, pH 7.4, and the column was subsequently washed with 20 ml of the equilibrium buffer. The rI1 eluted from the column with 0.02 M HEPES, 0.5 M NaCl, pH 7.4. Fractions containing recombinant thrombin were identified by amidolytic activity against S2238. Aliquots of each fraction (10 μl) were incubated with 90 μl of 0.5 mM S2238, and color development at 405 nm was monitored. Fractions containing S2238 activity were pooled and stored at -4 °C until used (within 5 days). The isolated rI1 was migrated as a single band (nonreduced) on NaDodSO$_4$-polyacrylamide minigels, which comigrated with thrombin prepared from human plasma.

**ActiveSiteTitration**—Concentrations of rI1, rMZa, and rMZdesF1a were determined by titrating an aliquot of the protein (1.6 ml, ~100 ng) with 5 μM PPACk in the presence of 200 mM EDTA, while measuring the fluorescence energy transfer from the proteases to DAPA ($\lambda_{em}$ 280 nm, $\lambda_{ex}$ 545 nm). Reaction of the protease with PPACk displaced DAPA from the active site resulting in a decrease in fluorescence intensity. The decrease was linear with respect to the concentration of PPACk, and extrapolation to the baseline fluorescence value yielded the active site concentration. The PPACk stock was dissolved shortly prior to use, and the concentration provided by the manufacturer was assumed to be correct. Titration of wild-type thrombin using this method yielded equivalent results to a p-nitrophenyl-p-guanidinobenzoate determination of active site titer (15).

**Time Course of Proteolysis** at Arg$^{55}$—Stock solutions of rMZa at 25,50, and 100 μg/ml were prepared in 0.02 M HEPES, 0.15 M NaCl, 2 mM CaCl$_2$. Immediately prior to use, and 4, 8, 12, 16, 20, and 36 min after addition of a small aliquot of 0.5 M EDTA, pH 7.4, to chelate the Ca$^{2+}$ present in the solution, aliquots equivalent to 2.5 μg of protein were withdrawn and quenched with the addition of 2 volumes of 0.2 μM acetic acid. The samples were reduced to dryness using a Savant Speed Vac, dissolved in gel loading buffer, and analyzed on NaDodSO$_4$-polyacrylamide gradient (5–15%) minigels under reducing conditions (16). After staining with Coomassie Blue, the gels were destained and scanned with an LKB 2202 laser densitometer, and the traces were subjected to gravimetric analysis. For this experiment and subsequent experiments where EDTA was added to buffer solutions containing Ca$^{2+}$, the pH of an equivalent mixture was monitored to ensure that the pH of the solution did not significantly change upon chelation of Ca$^{2+}$.

**Calcium Ion Modulation of Meizothrombin Activity**—A series of microcentrifuge tubes were prepared containing 0.02 M HEPES, 0.15 M NaCl, pH 7.4, with calcium ion concentrations ranging from 0 (1 mM EDTA, pH 7.4) to 5 mM CaCl$_2$. A fixed amount (2.5 μg) of rMZa or R155A was added to each tube, resulting in solutions with varying Ca$^{2+}$ concentrations with a minimum of 0.37 mM. The samples were then incubated for 1 h.
Calcium Ion Modulation of Meizothrombin Activity

RESULTS

Isolation and Characterization of Recombinant Proteins—Recombinant prothrombin and the variants rMZ and rMZdesF1 were expressed in stably transfected lines of BHK cells. The prothrombin variants rMZ and rMZdesF1 were isolated and were homogeneous as judged by analysis of Coomassie Blue-stained NaDodSO4-polyacrylamide gradient minigels. The proteins were activated by the snake venom protease dade difluoride membrane, amino-terminal sequence analysis of the prothrombinase complex at 4°C (6) and is stable when purified for 6 months at -20°C in 50% glycerol, storage of the purified protein in the absence of Ca2+ yielded rapid conversion to a species with the activity of the F1.2A chain visible on denaturing gels (Fig. 3). The rate of cleavage is not affected by equimolar concentrations of bovine F1 or a recombinant human prothrombin variant lacking factor Xa and thrombin cleavage sites (data not shown) nor are these species cleaved during the time course of the experiment. Although the concentration range studied was limited for technical reasons, modelling the meizothrombin proteolysis as an intermolecular reaction occurring with high (≥10 μM), intermediate (1 μM), or low (<1 μM) affinity using Kinsim (17), failed to produce a reasonable fit to the data, whereas modelling the reaction as an intramolecular event (first order) produced an excellent global fit to the data (Fig. 3). These results indicate that the cleavage at Arg[55] is an intramolecular rather than intermolecular event.

Calcium Ion Requirement for Maintenance of Intact rMZa—Samples of rMZa were incubated in buffer containing varying levels of Ca2+ for 60 min, and the extent of cleavage at Arg[55] was analyzed by NaDodSO4-polyacrylamide gel electrophoresis. Since the data in Fig. 3 clearly indicated that cleavage followed first order kinetics, the single time point measurements obtained in this experiment were used to calculate rate constants for cleavage according to the equation ln[M] = αt where [M0] and [M] are the levels of intact rMZa at time = 0 and time = t respectively. Analysis of the rate constants for cleavage (Fig. 4) revealed that the minimal Ca2+ concentration required for stability was approximately 1 mM, with cleavage occurring rapidly below this concentration. To account for the possibility that rMZa could be proteolyzed in subsequent experiments to rMZa* during incubation at low calcium ion concentrations, rMZa* was isolated and included as a control.

Metal Ion Dependence of rIIa, rMZa, rMZa*, and rMZdesF1a Activity against the Amidolytic Substrate S2238—Initial rates of cleavage indicated that rMZa has a half-life in excess of 28 days in the presence of Ca2+, but only 8 min in the absence of Ca2+. It is highly unlikely that this cleavage is a result of a trace contaminant in the bovine F1 or rMZa. Although the concentration range studied was limited for technical reasons, modelling the meizothrombin proteolysis as an intermolecular reaction occurring with high (≥10 μM), intermediate (1 μM), or low (<1 μM) affinity using Kinsim (17), failed to produce a reasonable fit to the data, whereas modelling the reaction as an intramolecular event (first order) produced an excellent global fit to the data (Fig. 3).

The data presented suggest that the cleavage at Arg[55] is an intramolecular rather than intermolecular event.
Calcium Ion Requirement for Maintenance of Intact rMZa. Aliquots of rMZa (1 mM) in 0.02 M HEPES, 0.15 M NaCl, pH 7.4, containing various concentrations of calcium ion. After a 60-min incubation at 22°C, the samples were quenched and analyzed as described in the legend for Fig. 3. From the determinations of the amount of rMZa remaining intact, the rate constants for cleavage at the varying Ca\(^{2+}\) concentrations were determined. Calculated half-lives are observed for rMZa and its cleaved variant (rMZa*), however, suggest that either the GlA domain or the F1 kringle interacts with divalent metal ions and thus affects the catalytic efficiency of the protease domain. The change in rates of S2238 hydrolysis by rMZa and rMZa* in the presence of divalent metal ions can be attributed to alteration of the k\(_{cat}\) toward S2238. K\(_{m}\) values for rMZa toward S2238 remain unchanged (1.9 ± 0.2 versus 2.2 ± 0.2 \(\mu\)M) in the presence or absence of 5 mM CaCl\(_2\), whereas the k\(_{cat}\) increases from 65 ± 3 s\(^{-1}\) in the absence of Ca\(^{2+}\) to 105 ± 5 s\(^{-1}\) in the presence of Ca\(^{2+}\).

Hydrolysis of the Esterolytic Substrate TAME by rMZa in the Presence and Absence of Ca\(^{2+}\)—Effects similar to those observed with S2238 hydrolysis are observed with TAME hydrolysis. When monitored by absorbance of the product at 247 nm, cleavage of this ester substrate is enhanced in the presence of Ca\(^{2+}\) with the turnover number increasing from 29 ± 0.9 s\(^{-1}\) to 37 ± 1.2 s\(^{-1}\) in the presence of 5 mM CaCl\(_2\).
Calcium Ion Modulation of Meizothrombin Activity

Previous studies of the binding of prothrombin and its activation intermediates to hirudin tail peptides (19), thrombomodulin, and exosite antibodies (20), have suggested that the fragment 1 domain may in some way block access to the primary anion binding exosite. Through measurements of the Ca$^{2+}$ dependence of meizothrombin autoproteolysis at Arg$^{55}$, activity toward a variety of substrates, and energy transfer to the active site probe DAPA, this study provides further evidence for interactions between the fragment 1 and protease domains of meizothrombin.

Cleavage of prothrombin at Arg$^{55}$ has previously been reported following lengthy incubation of prothrombin with thrombin in the absence of Ca$^{2+}$ (5). In the present studies, however, the cleavage of rMZA at Arg$^{55}$ follows first order kinetics, with the time course of the reaction being identical at three different concentrations of rMZA, indicative of an intramolecular cleavage event. These results imply a direct interaction between the amino-terminal portion of the fragment 1 domain with the active site of meizothrombin. Cleavage of rMZA at Arg$^{55}$ occurs rapidly in the absence of Ca$^{2+}$ with a half-life under 10 min, but is profoundly attenuated in the presence of Ca$^{2+}$.

Structural changes in the fragment 1 domain of prothrombin have been shown to accompany divalent metal ion binding by a number of methods including sedimentation velocity (21), intrinsic fluorescence (21), circular dichroism (22), and immunologic techniques (23). Due to the sensitivity of cleavage at Arg$^{55}$ to levels of Ca$^{2+}$, and the location of the cleavage site within the fragment 1 domain, this event is likely modulated by metal ion binding in the Gla domain. Indeed, cleavage of prothrombin by thrombin at Arg$^{55}$ is also inhibited in the presence of Ca$^{2+}$, and the protective effect has been attributed to conformational changes mediated by Ca$^{2+}$ binding to the Gla domain (24).

Although the three-dimensional structures for the apo- and Ca$^{2+}$-bound forms of human fragment 1 are not available, those of bovine fragment 1 in the absence and presence of Ca$^{2+}$ have been determined (25, 26). The structure of the Gla domain is disordered in the absence of Ca$^{2+}$, but the disulfide loop containing the cleavage site at Arg$^{55}$ is defined in both structures. Interestingly, the region around Arg$^{55}$ changes significantly upon Ca$^{2+}$ binding, with a trans to cis conformational change at Pro$^{54}$ and a 90° rotation of Arg$^{55}$ relative to its neighboring residues, enabling the formation of new salt links between Arg$^{55}$ and Gla$^{55}$, Leu$^{59}$, and Gla$^{60}$. These changes at the Arg$^{55}$ cleavage site likely explain the insensitivity of Ca$^{2+}$-bound meizothrombin to autoproteolysis. Another contributing factor to the stability of rMZA in the presence of Ca$^{2+}$ may be the disruption of interactions between the negatively charged residues of the Gla domain with positively charged portions of the protease domain (possibly the primary anion binding exosite) upon Ca$^{2+}$ binding. In any case, autocatalytic cleavage of rMZA at Arg$^{55}$ necessitates direct interaction between the fragment 1 and protease domains.

In addition to preventing autoproteolysis of rMZA, Ca$^{2+}$ increases the activity of rMZA toward small ester (TAME) and amide (S2238) substrates as well as increasing its fibrinogen clotting activity. Equivalent increases in activity are obtained with rMZA*, a species cleaved at Arg$^{55}$, so the increase in activity cannot be simply attributed to a Ca$^{2+}$-mediated protection from autoproteolysis. Neither rMZdesF1a, lacking the active site environment upon exposure to Ca$^{2+}$, were added to the wells of a microtiter plate containing varying concentrations of rIIa, rMZa, rMZdesF1a, and rMZdesF1a relative to rIIa. Fixed concentrations of rIIa (1 nM), rMZa (15 nM), or rMZdesF1a (3.6 nM) to give approximately equal clotting times in the presence of 5 mM CaCl$_2$ were added to the wells of a microtiter plate containing varying concentrations of EDTA to give a range of Ca$^{2+}$ concentrations (0–5 mM). Fibrinogen cleavage was initiated with the addition of 2 mg/ml fibrinogen in 0.02 M HEPES, 0.15 M NaCl, 0.01% Tween 80, pH 7.4, and the time to onset of clotting was determined by monitoring turbidity at 320 nm. Rates of fibrin formation relative to rIIa are plotted for rMZA (*), rMZdesF1a (●), and rMZdesF1a (○). The clot times for all species (rIIa, rMZa, rMZdesF1a, and rMZdesF1a) were titered with a solution of CaCl$_2$, while DAPA fluorescence was continuously monitored (λ$_{em}$ 280 nm, λ$_{ex}$ 545 nm). The results are expressed as a percentage of the original fluorescence signal.
fragment 1 domain, nor thrombin demonstrate this Ca$^{2+}$-dependent increase in activity, suggesting that interactions of Ca$^{2+}$ with the fragment 1 domain are responsible for the altered activity of rMZa.

Similar effects on the activity of rMZa are observed with both small substrates (amide and ester) and fibrinogen, although fibrinogen binding involves considerably more complex interactions with the enzyme than do the small substrates. Thus, obstruction of the active site or reduced turnover for some other reason, rather than a change in substrate binding, is responsible for the reduced activity of rMZa in the absence of Ca$^{2+}$. This interpretation is supported by the observation that values obtained for the $K_{c}$ for S2238 hydrolysis remain constant (1.9 versus 2.2 $\mu$M) in the absence or presence of 5 $\mu$M Ca$^{2+}$, while $K_{c}$ values change (65 versus 105).

Titration of rMZa with Ca$^{2+}$ in the presence of the reversible fluorescent inhibitor DAPA results in a 30% decrease in fluorescence energy transfer, with a half-maximal effect occurring at 0.5 $\mu$M Ca$^{2+}$. This result is similar to that obtained with meizothrombin prepared by activation of prothrombin with ecarin in the presence of dansyl-glutamylglycylarginyl chloromethyl ketone, yielding an active site blocked species with a catalytic activity toward small ester (TAME) and small and large amide substrates (S2238, fibrinogen). Interestingly, the transition for these effects borders on the range of free Ca$^{2+}$ in the plasma (1–1.3 $\mu$M) (31). Finally, these results are indicative of flexibility in the structure of prothrombin, allowing for association between the amino-terminal fragment 1 and carboxy-terminal serine protease domains.

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J. Biol. Chem. 1996, 271:8062-8067.
doi: 10.1074/jbc.271.14.8062

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