Elements of the Primary Structure of Thrombomodulin Required for Efficient Thrombin-activable Fibrinolysis Inhibitor Activation*

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Deletion and point mutants of soluble thrombomodulin were used to compare and contrast elements of primary structure required for the activation of thrombin-activable fibrinolysis inhibitor (TAFI) and protein C. The smallest mutant capable of efficiently promoting TAFI activation contained residues including the c-loop of epidermal growth factor-3 (EGF3) through EGF6. This mutant is 13 residues longer than the smallest mutant that functioned well with protein C; the latter consisted of residues from the interdomain loop connecting EGF3 and EGF4 through EGF6. Alanine point mutants showed no loss of function in protein C activation for mutations within the c-loop of EGF3. In TAFI activation, however, alanine mutations cause a 50% reduction at Tyr-337, 67% reductions at Asp-338 and Leu-339, and 90% or greater reductions at Val-340, Asp-341, and Glu-343. A mutation at Asp-349 in the peptide connecting EGF3 to EGF4 eliminated activity against both TAFI and protein C. Oxidation of Met-388 in the peptide connecting EGF5 to EGF6 reduced the rate of protein C activation by 80% but marginally, if at all, affected the rate of TAFI activation. Mutation at Phe-376 severely reduced protein C activation but only marginally influenced that of TAFI. A Q387P mutation, however, severely reduced both activities. TAFI activation was shown to be Ca\(^{2+}\)-dependent. The response, unlike that of protein C, was monotonic and was half-maximal at 0.25 mm Ca\(^{2+}\). Like protein C activation, TAFI activation was eliminated by a monoclonal antibody directed at the thrombin-binding domain (EGF5) but was not affected by one directed at EGF2. Thus, elements of structure in the thrombin-binding domain are needed for the activation of both protein C and TAFI, but more of the primary structure is needed for TAFI activation. In addition, some residues are needed for one of the reactions but not the other.

Thrombin-activable fibrinolysis inhibitor (TAFI)\(^1\) is a 60-kDa plasma protein that circulates in plasma at concentration of about 75 nm (1). It is a zymogen that is activated to a carboxypeptidase B-like enzyme by a single thrombin-catalyzed cleavage at arginine 92 (2–4). The enzyme, designated TAFI\(_a\), catalyzes removal of carboxyl-terminal arginine and lysine residues in fibrin as it undergoes fibrinolysis (5). As a consequence, feedback up-regulation of plasminogen activation is eliminated, and the process of fibrinolysis is suppressed. The activation of TAFI by thrombin and subsequent actions of TAFI\(_a\) define a molecular connection between the coagulation and fibrinolytic cascades, such that activation of the former suppresses activity in the latter (6). Although thrombin at the high levels generated after the clotting of fibrin occurs can activate sufficient TAFI to suppress fibrinolysis, thrombin by itself is a relatively weak activator (2, 7). Thrombin bound to thrombomodulin, however, activates TAFI with a catalytic efficiency 1250-fold greater than that of thrombin alone (6). Thus, the thrombin-thrombomodulin complex is probably the physiologic activator of TAFI.

Thrombomodulin is an intrinsic membrane protein of 557 amino acid residues located on the luminal side of the endothelium (8, 9). It possesses a domain structure comprising a lectin-like domain (residues 1–226), six tandem epidermal growth factor-like domains joined by small interdomain peptides (EGF1-(227–262), EGF2-(270–305), EGF3-(311–344), EGF4-(351–386), EGF5-(390–407), EGF6-(427–462)), a serine/threonine-rich domain (residues 469–497), a transmembrane domain (residues 498–521) and an intracellular domain (residues 522–557). It was discovered in a search for a cofactor for the thrombin-catalyzed activation of the zymogen, protein C, to the anticoagulant enzyme, activated protein C (10). Like TAFI activation, protein C activation by thrombin is stimulated about 1200-fold by thrombomodulin (11). The activation of protein C by the thrombin-thrombomodulin complex defines a negative feedback loop in the coagulation cascade that down-regulates thrombin formation and is essential for the proper regulation of fibrin formation (8, 9).

Structure/function studies of protein C activation indicate that thrombin binding is mediated through the EGF5 and EGF6 domains of thrombomodulin (12, 13). Thrombin binding, however, is not sufficient to stimulate protein C activation. Further elements of primary structure required include the EGF4 domain and the six-residue peptide that connects EGF3 to EGF4 (14, 15). In addition, methionine 388, which is on the loop connecting EGF5 to EGF6, appears important in protein C activation because when it is oxidized 90% loss of function occurs (16). This residue, however, can be replaced with leucine with a 2-fold gain in function (17). Alanine-scanning mutagenesis of the EGF-like domains of thrombomodulin between residues 333 and 462 indicates that replacing residues Glu-357, Tyr-358, and Phe-376, in EGF4, or Lys-349, in the peptide connecting EGF4 to EGF3, with alanine severely diminishes the activity of thrombomodulin in protein C activation (18). Critical residues in EGF-like domains 5 and 6 were also iden—

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1 The abbreviations used are: TAFI, thrombin-activable fibrinolysis inhibitor; EGF, epidermal growth factor; DAPA, dansylarginine N-(3-ethyl-1,5-pentanediyl)amide; FAAK, furfuryl(acryloyl)-alaninyl-lysine.
tified. In all, of 77 residues mutated, 22 were found to be critical for TM function in protein C activation.

In some respects, thrombomodulin appears to stimulate thrombin-catalyzed activation of protein C and TAFI in very similar ways. The magnitude of the increases in catalytic efficiency are about the same with bothzymogens (6, 11). The kinetics of activation of bothzymogens can be accurately described by a mechanism in which thrombin can interact with either thrombomodulin or the substrate, and the so-formed binary complexes can interact with the third component to form a ternary thrombin-thrombomodulin-substrate complex, from which either activated protein C or TAFIa are generated.

Differences in the two reactions also have been observed. For example, the P4–P4’ sequences around the activating cleavage site in protein C and TAFI are different. That of protein C is VDPR↓LIDG, whereas that of TAFI is VSPR↓ASAS (2, 19).

Thus, the paradigm whereby protein C is a poor substrate for thrombin in the absence of thrombomodulin because of the negatively charged groups at positions P3 and P3’ (20) does not apply to TAFI activation. In addition, Kokame et al. (21) showed that TAFI activation by cell-bound thrombomodulin constructs is supported well when EGF3 is included but not in its absence, thereby demonstrating that elements of EGF3 are required for this reaction. Preliminary work by our group has shown that the minimal structure required for protein C activation, which comprises EGF domains 4–6 plus the six residues connecting EGF4 to EGF3, does not function well in TAFI activation (15), but inclusion of the c-loop of EGF3 provides a structure capable of supporting TAFI activation as well as full-length thrombomodulin.

The studies reported here were carried out in order to identify more clearly the elements of structure required for TAFI activation and to compare TAFI activation with protein C activation. The results generally show that, although the two processes require common elements of thrombomodulin structure, differences exist such that more of the primary structure of thrombomodulin is required for TAFI activation than for protein C activation, and some residues are needed in one reaction but not the other.

**EXPERIMENTAL PROCEDURES**

**Proteins and Reagents**—Truncated forms of thrombomodulin comprising Solulin (residues 4–490), TMα (residues 227–462), TMβ-c-loop 3–6 (residues 353–462), and TMβ4–6 (residues 345–362) were prepared as described previously (14). SD9 cells were transfected with the TM constructs, and the proteins were isolated from the media by a combination of chromatography procedures utilizing anion exchange, gel filtration, and thrombin affinity. Purity, assessed by SDS-polyacrylamide gel electrophoresis and silver staining, was 95% or greater.

Thrombomodulin and TAFI Activation—The effects of thrombomodulin variants on TAFI activation were measured by incubating TAFI and thrombin at fixed concentrations with the thrombomodulin variants for 10 min at room temperature in HBS plus 5.0 mM CaCl2. The final concentrations of TAFI and thrombin were 0.5 μM and 1.0 nM, respectively.

The thrombomodulin concentrations ranged from 0 to 100 nM. The incubations were carried out in the wells of a microtiter plate, and the volume was 20 μl. The reactions were stopped by adding 180 μl of a solution containing DAPA and FAAK in HBS. The final concentrations of DAPA and FAAK were 27 and 500 μM, respectively. The time course of absorbance at 340 nm was then measured to calculate the TAFI concentrations and thus the rates of TAFI activation. In order to measure the effects of the thrombomodulin variants on protein C activation, thrombin, human protein C, and the thrombomodulin variants were incubated for 10 min in HBS, 5.0 mM CaCl2, in a volume of 50 μl in the wells of a microtiter plate thermostatted at 37 °C. The final concentrations of protein C and thrombin were 1.0 μM and 2.0 nM, respectively, and the thrombomodulin concentration was varied from 0 to 100 nM. The thrombin concentrations ranged from 0 to 100 nM. The incubations were carried out in the wells of a microtiter plate and the reaction volume was 20 μl. The reactions were stopped by adding a solution (150 μl) of DAPA, FAAK, and the synthetic parenteral substrate FAKK. The absorbance was monitored over time at 405 nm, and the rate of change of absorbance was determined in order to calculate the activated protein C concentrations and hence calculate the rates of protein C activation.

Measurement of the Rates of Protein C and TAFI Activation with Point Mutants of Thrombomodulin—For the activation of TAFI, a 20-μl aliquot of each periplasmic extract was preincubated with thrombin (13 nM final) in 20 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM CaCl2 for 5 min at room temperature. The mixtures were then incubated with purified recombinant TAFI (18 nm final) and a substrate, hippuryl-arginine (1.0 mm final), in a total volume of 60 μl for 60 min. The amount of activated TAFI was quantitated by measuring the hydrolysis of hippuryl-arginine to hippuric acid, followed by conversion of hippuric acid to a chromogen with 80 μl of phosphate buffer (0.2 μl, pH 8.3) and 60 μl of 3% cyanuric acid in dioxane (w/v). After thorough mixing, absorbance of the clear supernatant was measured at 382 nm. The amount of thrombin-dependent activation of TAFI was calculated by subtracting the background absorbance produced in the absence of thrombin for each mutant.

Activation of protein C by TMα(M388L) alanine mutants was assayed as described previously (16). Assays contained extracts of DH5α cells transfected with either pSelect-1 vector (no TMα), wild-type TM(388), or TMα(M388L) as internal controls. Cofactor activities of TMα(M388L) alanine mutants were expressed as percentages of the activity of TMα(M388L). Each TM mutant was assayed for both protein C and TAFI activation in duplicate using three independent preparations of extracts.

**Measurement of the Effect of Anti-thrombomodulin Antibodies on TAFI Activation**—Monoclonal antibodies to the EGF2 or EGF5 domains of thrombomodulin were incubated with TMα at room temperature for 60 min in 0.02 mM HEPES, 0.15 mM NaCl, 0.01% Tween 80, pH 7.4 (HBS), for 60 min in the wells of a microtiter plate. The volume was 3.5 μl; the thrombin concentration was 300 nM, and the antibody concentrations ranged from 0 to 1380 nM. The volume was then increased to 10 μl by adding 6.7 μl of a solution of thrombin and Ca2+ in HBS, and another 10 μl of a solution of TAFI was added. The final volume was 20 μl, and the final concentrations of Ca2+, thrombin, and TAFI were 10 mM, 2 mM, and 500 nM. The solution was incubated for 10 min to allow for partial TAFI activation, and then a solution (180 μl) containing FAAK (furanyl-(acyrroyl)-alaninyl-lysine, Sigma) and DAPA was added. The final concentrations of FAAK (TAFIα substrate) and DAPA (thrombin inhibitor) were 500 and 27 μM, respectively. Activated TAFIα levels were then deduced by monitoring the absorbance at 340 nm over time.
(2-fold in the case of 100 mM chloramine T) and incubating an additional 30 min. The ability of the oxidized samples to support protein C activation was determined by diluting them 10-fold in a solution of CaCl2 (5 mM), protein C (10 nM), and thrombin (1 nM). The final volume was 100 μl. After further incubation for 5 min, protein C activation was quenched by adding a solution (100 μl) of EDTA (20 mM), DAPA (60 μM), and S2366 (400 μM). The activated protein C level was then measured by monitoring the absorbance at 405 nm in a microtiter plate reader. The ability of the oxidized thrombomodulin variant to potentiate TAFI activation was measured by diluting it 10-fold in the above-described solution containing TAFI (1 μM) in place of protein C. After 5 min at room temperature, the reaction was quenched by adding a solution (100 μl) of FAAK (200 μM) and β-phenylalaninylarginyl chloromethyl ketone (4 μM). The absorbance was then monitored over time at 324 nm to measure the level of TAFIa.

RESULTS

The Effect of Anti-thrombomodulin Monoclonal Antibodies on TAFI Activation—Two monoclonal antibodies directed, respectively, at the EGF2 and EGF5 domains of thrombomodulin (18) were examined for their ability to inhibit TAFI activation (Fig. 1). The antibody directed against EGF2 had no impact on function, but the one directed at EGF5 inhibited function by approximately 95% at a level of antibody stoichiometric with thrombomodulin. These data are very similar to those reported previously (14, 15). TM34–6 was the smallest construct able to support protein C activation at this level. In contrast, although Solulin and TM4–6 supported TAFI activation well, TM34–6, did not. Results obtained with these as well as other constructs in similar experiments are summarized in Table I. As these results indicate, a construct which includes EGF domains 4–6 plus the six residue peptide connecting EGF3 to EGF4 (residues 345–462) was found to be the smallest construct capable of supporting protein C activation.

FIG. 1. The effect of antithrombomodulin monoclonal antibodies on TAFI activation. Monoclonal antibodies directed against the EGF5 (○) and EGF2 domains (■) were included in TAFI activation reactions. The thrombomodulin concentration prior to assay for TAFIa was 100 nM.

The Role of Met-388 in TAFI Activation—Previous work showed that oxidation of Met-388 of thrombomodulin by chloramine T or H2O2 decreases cofactor activity for protein C activation by 90% or more (16). Further work showed that replacement of Met-388 with leucine not only protects thrombomodulin from oxidation but also doubles its activity in protein C activation (17). Because these studies identified Met-388 as a very important residue in protein C activation, similar experiments were performed to investigate the impact of oxidation of Met-388 on TAFI activation. A soluble construct of thrombomodulin comprising the c-loop of EGF3 through the EGF domain and containing Met-388 was treated with chloramine T. The excess chloramine T was reduced with methionine, and the cofactor activity was measured with TAFI and protein C as substrates (Fig. 2). Whereas oxidation reduced the activity with protein C by over 80%, it reduced activity with TAFI only marginally. In addition, whereas substitution of methionine with leucine at position 388 increased activity in protein C activation 2-fold, it had no effect on TAFI activation (Fig. 4B). These data imply that oxidation of M388 has less impact on TAFI activation than on protein C activation.

Thrombin-catalyzed Activation of TAFI and Protein C with Deletion Mutants of Thrombomodulin Truncated from the Amino Terminus—Initial rates of the activation of PC or TAFI were determined at various concentrations of forms of recombinant thrombomodulin truncated from the amino terminus. Examples are shown in Fig. 3, A and B, for thrombomodulin constructs including full-length soluble thrombomodulin (Solulin, residues 4–490); TM4–6, which consists of the six EGF domains of thrombomodulin (residues 227–462); and TM34–6, which consists of EGF domain 4 through 6 plus the six residue peptide connecting EGF3 to EGF4 (residues 345–462). All three constructs were approximately equivalent in protein C activation as has been shown previously (14, 15). TM34–6 was the smallest construct able to support protein C activation at this level. In contrast, although Solulin and TM4–6 supported TAFI activation well, TM34–6, did not. Results obtained with these as well as other constructs in similar experiments are summarized in Table I. As these results indicate, a construct which includes EGF domains 4–6 plus the interdomain loop between EGF domains 3 and 4 will efficiently promote protein C activation but not TAFI activation. With TAFI, further amino-terminal elements of structure of thrombomodulin associated with the c-loop of EGF3 are required.

TAFI Activation by Alanine Point Mutants of Thrombomodulin—In order to further identify residues within the c-loop of EGF3 required for cofactor activity in TAFI activation, point mutants with alanine substituted for individual amino acids contained within the thrombomodulin sequence spanning the c-loop of EGF3 through the interdomain loop connecting EGF domains 3 and 4 (residues 333–350) were prepared and investigated with respect to their abilities to support protein C and TAFI activation. The alanine point mutants of thrombomodulin were expressed as secretory proteins, and periplasmic extracts were prepared as described previously (18). In an earlier study, we established the conditions in which recombinant mutants in periplasmic extracts were used without purification to measure their relative cofactor activity for the activation of protein C. Briefly, the M388L mutant in periplasmic extracts had a protein C cofactor activity of 20.1 ± 5.2 units/ml (n = 18), and the ratio of M388L/Met-388 cofactor activities was 2.35 ±
0.39, with an inter-assay coefficient of variation of 16.7% (n = 18). Since total expression levels of M388L and Met-388 in extracts were similar by Western blot analysis using polyclonal anti-TME antibody, the ratio of M388L to Met-388 (wild type) activities in extracts was consistent with those of the purified proteins, showing that thrombomodulin mutants in the periplasmic extracts were correctly folded. This was confirmed when specific activities for Met-388 and M388L were determined by measuring antigen levels in extracts using an enzyme-linked immunosorbent assay for thrombomodulin with two monoclonal anti-TME antibodies. The specific activities of Met-388 and M388L were 437,000 ± 24,000 and 1,019,000 ± 87,000 units/mg of protein, respectively, giving a M388L/Met-388-specific ratio of 2.33 (n = 2). Furthermore, other mutants that exhibited very low cofactor activities for protein C were analyzed by Western blotting to ensure that the expression levels were similar to those of active mutants. Thus, this system provides a fast and easy method for comparing cofactor activities of different mutants. In all the experiments, we included extracts prepared from E. coli transformed with a vector only, Met-388 (wild type), and M388L as internal controls. In the current study, the ratio of M388L/Met-388 cofactor activities for activation of protein C was 2.0 ± 0.38 (n = 6).

The results obtained with these mutants (Fig. 4, A and B) indicate that all mutants within the c-loop of EGF3 had modest or negligible effects on protein C activation. These results are consistent with those obtained previously (18). In contrast, when the same periplasmic extracts containing these mutants were tested in a TAFI cofactor activity assay, several of the mutants substantially influenced TAFI activation. Because protein C activation was not significantly different from control values, it is likely that the proteins are in the correct conformation in the periplasmic extracts. With respect to TAFI activation, the mutation Y337A caused a 50% reduction in activity; those at Asp-338 and Leu-339 caused a 67% loss; and those at Val-340, Asp-341, and Glu-343 caused greater than 90% losses. The alanine substitution mutation at Val-345, Glu-346, or Val-348 within the interdomain loop between EGF3 and -4 caused modest 30–40%
reductions in protein C activation (Fig. 4B). Similar effects on TAFI activation were obtained with mutations E346A and V348A, whereas mutation V345A resulted in a 70% reduction in TAFI activation. The D349A mutation in the interdomain loop reduced both protein C and TAFI activation rates by 95%. Thus, these data correlate well with the results obtained with deletion mutants (Fig. 3 and B) in that they imply that the c-loop of EGF3 is not needed for protein C activation but is for TAFI activation. They also identify residues Tyr-337, Asp-338, Leu-339, and Val-345 as important for TAFI activation and residues Val-340, Asp-341, and Glu-343 as virtually essential. In addition, Asp-349 appears to be very important in the activation of both zymogens. We also investigated the effect of three mutations within EGF4, previously found to be critical for the activation of protein C (18), on TAFI activation. The mutations at Glu-357 and Tyr-358 (Fig. 4B) caused marked reductions in both TAFI activation and protein C activation. The mutation at Phe-376, in contrast, resulted in a profound loss in protein C activation but only in a modest reduction in TAFI activation. Another mutation, Q387P, which causes a large reduction of cofactor activity for protein C activation (17), also reduced cofactor activity for TAFI activation by over 95%.

**Ca**$$^{2+}$$ **Dependence of Thrombomodulin-mediated Protein C and TAFI Activation**—Others (25, 26) have shown that protein C activation in the presence of thrombin and thrombomodulin without chondroitin sulfate exhibits a complex, biphasic dependence on the Ca$$^{2+}$$ concentration. As the Ca$$^{2+}$$ concentration is increased, the rate of protein C activation typically increases up to a maximum at about 0.25 mM Ca$$^{2+}$$ and then decreases toward a plateau at about 5.0 mM Ca$$^{2+}$$. In order to determine whether this characteristic is shared by TAFI activation, Ca$$^{2+}$$ concentrations were systematically increased, and initial rates of TAFI activation in the presence of Solulin were measured. Similar experiments were performed with protein C activation for comparison. The results (Fig. 5, A and B) indicate that both protein C and TAFI activation reactions are Ca$$^{2+}$$-dependent. TAFI activation, however, does not exhibit the biphasic response of protein C activation. Instead, initial rates of TAFI activation are monophasic with respect to Ca$$^{2+}$$ and increase toward a maximum at levels of Ca$$^{2+}$$ exceed 1–2 mM. The half-maximal response is obtained at 0.24 ± 0.05 mM Ca$$^{2+}$$. The concentration of Ca$$^{2+}$$ when the rate of TAFI activation is one-half of its maximum is very similar to that at which the maximum is obtained in the biphasic Ca$$^{2+}$$ dependence of protein C activation.

**DISCUSSION**

Thrombomodulin is a cofactor for the thrombin-catalyzed activation of both protein C and TAFI. In both cases a single proteolytic cleavage generates an enzyme from a plasma zymogen, and both enzymes modulate the balance between fibrin formation and degradation. Activated protein C, which is generated from protein C, is a serine protease that down-regulates thrombin formation and thereby down-regulates fibrin formation. TAFI is activated to TAFIa, which is a carboxypeptidase B-like enzyme that suppresses plasmin formation and thereby attenuates fibrin removal. Consequently, thrombomodulin is involved in the regulation of both the coagulation and fibrinolytic cascades.

The present studies indicate that the activation of both protein C and TAFI requires the fifth and sixth EGF domains of thrombomodulin, which contain structures needed for thrombin binding and high affinity Ca$$^{2+}$$ binding (25). Although these elements of structure are necessary for function, they are not sufficient to enhance activation of either protein C or TAFI. With protein C as substrate, EGF4 plus the 6-residue peptide connecting it to EGF3 are also needed. Although these elements of structure are both necessary and sufficient for protein C activation, they are not sufficient for TAFI activation. Thus, further residues amino-terminal to the peptide connecting EGF3 to EGF4 are required. The current work showed that the 13 residues comprising the c-loop of EGF3 suffice to restore fully TAFI activation function when included with the minimum fragment necessary for protein C activation. This is consistent with the results reported previously by Kokame et al. (21) who showed that EGF3, in addition to EGF domains 4–6, is required for TAFI activation by cell-bound constructs of thrombomodulin.

Our current study has identified the important residues within the c-loop of EGF3 and the interdomain connecting it to EGF4 for activation of TAFI. The residues Val-340, Asp-341, Glu-343, and Asp-349 are essential for the activation of TAFI. Furthermore, the mutations at Tyr-337, Asp-338, Leu-339, Val-345, Glu-346, and Val-348 caused a significant reduction in the activation of TAFI. In addition, two residues, Glu-357 and Tyr-358, within EGF4, which were identified as critical residues for protein C activation, were shown to be important for TAFI activation as well. Intriguingly, the difference in importance of Phe-376 for TAFI and protein C activation suggests the requirements for thrombomodulin structure are more constrained when protein C is the substrate of the thrombin-
thrombomodulin complex.

Both TAFI activation and protein C activation by thrombin-thrombomodulin are Ca\(^{2+}\)-dependent. The dependences are quite different, however. Protein C activation rates exhibit a biphasic response to Ca\(^{2+}\) with a maximum at about 0.25 mM Ca\(^{2+}\). TAFI activation rates increase monotonically with Ca\(^{2+}\), and the half-maximal effect occurs at a Ca\(^{2+}\) concentration of 0.25 mM. A recent study by Light et al. (25) of the binding of Ca\(^{2+}\) to thrombomodulin identified one very high affinity site in EGF6 (K\(_{D}<2 \mu M\)) and another site (K\(_{D} = 30 \mu M\)), which most likely comprises the Ca\(^{2+}\)-binding consensus site in EGF3 (residues 307–338). The very high affinity site appears to be very important in thrombin binding. The role of the second site in protein C and TAFI activation, if any, is difficult to infer from the present data, even though both protein C and TAFI activation show responses to Ca\(^{2+}\) at levels that far exceed that required to saturate fully the very high affinity site. Since protein C activation is fully supported by a construct that required to saturate fully the very high affinity site. Since protein C activation is fully supported by a construct that required to saturate fully the very high affinity site. Since protein C activation is fully supported by a construct that required to saturate fully the very high affinity site. Since protein C activation is fully supported by a construct that required to saturate fully the very high affinity site. Since protein C activation is fully supported by a construct that required to saturate fully the very high affinity site.

Whether this thrombomodulin construct possesses a second Ca\(^{2+}\)-binding site is not known, but its amino terminus possesses six carboxyl-terminal residues of the 32-residue Ca\(^{2+}\)-binding consensus sequence. The Ca\(^{2+}\) dependences of protein C and TAFI activation reported here were both obtained with Solulin, which does have both Ca\(^{2+}\)-binding sites. The increase in protein C activation rates up to a maximum at 250 \(\mu M\) Ca\(^{2+}\) could reflect binding of Ca\(^{2+}\) to protein C, to thrombomodulin, or both. The half-maximal rate at 250 \(\mu M\) Ca\(^{2+}\) in TAFI activation most likely reflects an interaction of Ca\(^{2+}\) with thrombomodulin. Although the dissociation constant for one of Ca\(^{2+}\)-thrombomodulin interactions is 30 \(\mu M\), a half-maximal increase in rate 250 \(\mu M\) Ca\(^{2+}\) in protein C activation and the half-maximal effect in TAFI activation could represent similar Ca\(^{2+}\), thrombin, thrombomodulin, substrate interactions.

The differential effects of oxidation of Met-388 on protein C and TAFI activation have notable potential ramifications. They make possible a mechanism whereby protein C activation could be attenuated without influencing TAFI activation under physiologic or pathophysiologic conditions. As shown by Glaser et al. (27) but can be rescued by homologous recombination to

The results of Hall et al. (29) complement those reported here on different structural requirements in thrombomodulin for protein C and TAFI activation. Both previous and current results indicate that the interaction of the thrombin-thrombomodulin complex with TAFI differs from that with protein C at the level of both the enzyme, thrombin, and its cofactor, thrombomodulin.