Rational Design of a Potent Anticoagulant Thrombin* 

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Angelene M. Cantwell and Enrico Di Cera‡
From the Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110

Thrombin acts as a procoagulant when it cleaves fibrinogen and promotes the formation of a fibrin clot and functions as an anticoagulant when it activates protein C with the assistance of the cofactor thrombomodulin. The dual function of thrombin in the blood poses the challenge to turn the enzyme into a potent anticoagulant by selectively abrogating fibrinogen cleavage. Using functional and structural data, we have rationally designed a thrombin mutant, W215A/E217A, that cleaves fibrinogen with a value of $K_{\text{cat}}/K_m$ about 20,000-fold slower than wild-type but activates protein C in the presence of thrombomodulin with a specificity comparable with wild-type. This mutant demonstrates for the first time that the relative specificity of thrombin toward fibrinogen and protein C can be completely reversed.

Thrombin plays two important and opposing functions in the blood (1). It acts as a procoagulant when it converts fibrinogen into an insoluble fibrin clot that anchors platelets to the site of lesion and initiates processes of wound repair, and it acts as an anticoagulant when it activates protein C with the assistance of the endothelial receptor thrombomodulin. Binding of thrombomodulin competitively suppresses the ability of thrombin to cleave fibrinogen (2) or the platelet receptor PAR1 (3) but enhances >1,000-fold the specificity of the enzyme toward the zymogen protein C. Activated protein C cleaves and inactivates factors Va and VIIIa, two essential cofactors of coagulation, thereby down-regulating both the amplification and progression of the coagulation cascade (4). Scavenging of thrombin by thrombomodulin and activation of protein C in the microcirculation constitute the natural anticoagulant pathway that prevents massive intravascular conversion of fibrinogen into an insoluble clot upon thrombin generation (2). In addition, thrombin is irreversibly inhibited at the active site by the serine protease inhibitor antithrombin III with the assistance of heparin (5).

The structural determinants of thrombin-fibrinogen interaction are known in sufficient detail (6), but there is still considerable uncertainty on how thrombin recognizes protein C at the molecular level. Recent crystallographic data on the thrombin-thrombomodulin complex have revealed little changes induced by thrombomodulin on the conformation of thrombin (7), further validating the thesis from previous functional studies that the thrombomodulin-induced enhancement of protein C activation is because of an effect of the cofactor on protein C and not on thrombin (8).

Considerable interest has recently emerged on the possibility of dissociating the procoagulant and anticoagulant activities of thrombin (1). Fibrinogen binding to thrombin requires the integrity of exosite I, together with the active site region (9). Protein C activation by thrombin also requires integrity of exosite I, because this is the locale for thrombomodulin binding (9). In the active site region, fibrinogen and protein C make similar, though not identical, contacts with the enzyme. This has made it possible to differentially affect substrate recognition using Ala mutants of key residues of thrombin. A systematic Ala scan of thrombin residues has been carried out by Tsang et al. (10, 11). In this study, more than 70 Ala mutants of solvent-exposed residues were characterized in their interaction with fibrinogen, protein C, and antithrombin III. A striking discovery emerged from these studies was that the balance between procoagulant and anticoagulant activities of thrombin, measured respectively as the ability to cleave fibrinogen or to activate protein C in the presence of thrombomodulin, could be altered substantially by mutation of residue Glu217 (12). The E217A mutant has reduced activity toward fibrinogen 40-fold and compromised protein C activation of only 2-fold and shows a modest but significant anticoagulant effect in vivo (12). Following this observation, other Ala mutations of thrombin residues in the Na+ binding environment have been reported to produce anticoagulant thrombins by shifting the equilibrium toward the anticoagulant slow form of the enzyme (13, 14). Although these mutations enhance the anticoagulant properties of thrombin, they reduce but do not abrogate cleavage of fibrinogen. A potent anticoagulant thrombin should have practically no clotting activity, should retain protein C activation in the presence of thrombomodulin, and should be inactivated only marginally by the natural inhibitor antithrombin III to ensure a prolonged lifetime in the blood (1, 14). Previous studies have indicated that the pursuit of this goal is feasible (12–14). Here we demonstrate that such a potent anticoagulant thrombin can be engineered rationally by combining mutations around the active site that individually reduce fibrinogen binding but not protein C activation.

MATERIALS AND METHODS

Site-directed mutagenesis of human α-thrombin was carried out in a HPC4-pNUT expression vector, using the Quickchange site-directed mutagenesis kit from Stratagene. Expression of mutant (W215A, E217A, and W215A/E217A) and wild-type thrombins was carried out in baby hamster kidney cells as described previously (15). The enzyme was activated with the prothrombinase complex for 30 min at 37 °C or with the immobilized snake venom enzyme ecarin. Activated thrombin was purified to homogeneity by fast protein liquid chromatography using Resource Q and S columns with a linear gradient from 0.05 to 0.5 M choline chloride, 5 mM MES, pH 6, at room temperature. Mutants were checked for incomplete activation and/or autolytic digestion by N-terminal amino acid sequencing. Electrospray mass spectrometry yielded

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‡ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biophysics, Washington University School of Medicine, Box 8231, St. Louis, MO 63110. Tel.: 314-362-4185; Fax: 314-362-7183; E-mail: enrico@caesar.wustl.edu.

1 The abbreviations used are: MES, 4-morpholineethanesulfonic acid; PEG, polyethylene glycol.
Experimental conditions are 5 mM Tris, 0.1% PEG, 145 mM NaCl, pH 7.4, 37 °C. Values for the W215A mutant, with the exception of the activation of protein C in the absence of thrombomodulin, are from Ref. 17. The value of $\Delta G_s$, in kcal/mol, was calculated according to Equation 1 in the text, using the values of $s = k_{cat}/K_m$ for wild-type and mutants given in the table. WT, wild-type; TM, thrombomodulin; RAP, relative anticoagulant potency.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>W215A</th>
<th>E217A</th>
<th>W215A/E217A</th>
<th>$\Delta G_s$</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-D-Phe-Pro-Arg-p-nitroanilide</td>
<td>97 ± 5</td>
<td>0.91 ± 0.01</td>
<td>3.8 ± 0.3</td>
<td>0.0028 ± 0.0001</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>17 ± 1</td>
<td>0.034 ± 0.002</td>
<td>0.27 ± 0.02</td>
<td>0.00089 ± 0.00007</td>
<td>-0.3 ± 0.1</td>
</tr>
<tr>
<td>Fibrin</td>
<td>8.1 ± 0.5</td>
<td>0.053 ± 0.003</td>
<td>0.15 ± 0.02</td>
<td>0.0021 ± 0.0001</td>
<td>-0.5 ± 0.1</td>
</tr>
<tr>
<td>Protein C – TM</td>
<td>150 ± 10</td>
<td>1.0 ± 0.01</td>
<td>15 ± 1</td>
<td>0.57 ± 0.03</td>
<td>-1.1 ± 0.1</td>
</tr>
<tr>
<td>Protein C + TM</td>
<td>220 ± 10</td>
<td>75 ± 6</td>
<td>140 ± 10</td>
<td>33 ± 2</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>PAR1</td>
<td>26 ± 1</td>
<td>1 ± 0.1</td>
<td>0.66 ± 0.01</td>
<td>0.026 ± 0.001</td>
<td>0.0 ± 0.1</td>
</tr>
<tr>
<td>Antithrombin III</td>
<td>13 ± 1</td>
<td>0.56 ± 0.04</td>
<td>1 ± 0.1</td>
<td>0.0040 ± 0.0003</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>$k_{cat}$ (µM⁻¹·s⁻¹)</td>
<td>1</td>
<td>170 ± 20</td>
<td>40 ± 3</td>
<td>2800 ± 300</td>
<td></td>
</tr>
</tbody>
</table>

a In the absence of rabbit thrombomodulin but in the presence of 5 mM CaCl₂.
b In the presence of 100 nM rabbit thrombomodulin and 5 mM CaCl₂.
c In the presence of 0.5 USP units/mL of heparin.
d Relative anticoagulant potency calculated as the ratio of the rate for protein C activation over the rate for fibrinopeptide A release, relative to the same ratio of wild-type thrombin (14).

molecular weights consistent with the mutations introduced and indicated identical glycosylation between wild-type and mutant constructs. The active site concentration was determined by titration with hirudin and was found to be >95% in all cases.

All assays were carried out under experimental conditions of 5 mM Tris, 0.1% PEG, 145 mM NaCl, pH 7.4, 37 °C. The chromogenic substrates H-D-Phe-Pro-Arg-p-nitroanilide specific for thrombin and H-D-Asp-Arg-Arg-p-nitroanilide specific for activated protein C were purchased from Midwest Bio-Tech (Carmel, IN). The values of $k_{cat}/K_m$ and $k_{cat}$ were obtained from the analysis of progress curves of the release of p-nitroaniline (measured at 405 nm) as a function of substrate concentration taking into account product inhibition when present. The interaction of thrombin with fibrinogen and fibrin was studied in terms of the release of fibrinopeptides A and B as described (16). The interaction of thrombin with PAR1 was studied from the kinetics of cleavage of a soluble fragment corresponding to the extracellular portion of the receptor as detailed elsewhere (17). The inhibition of thrombin by antithrombin III in the presence of heparin and the activation of protein C in the presence or absence of rabbit thrombomodulin were carried out and analyzed as described (12).

The coupling between the mutations of Trp215 and Glu217 was quantified using the coupling free energy (18),

$$\Delta G_s = -RT \ln \left( \frac{S_{W215A/E217A}}{S_{WT/WT}} \right)$$

where $s$ refers to the value of $k_{cat}/K_m$, $R$ is the gas constant, and $T$ is the absolute temperature. The value of $\Delta G_s$ reflects interactions between the individual mutations that either enhance ($\Delta G_s < 0$) or reduce ($\Delta G_s > 0$) the specificity in the double mutant beyond simple additivity ($\Delta G_s = 0$). A value of $\Delta G_s > 0$ also means that the single mutations are positively coupled (or act synergistically) in reducing specificity in the double mutant, whereas a value of $\Delta G_s < 0$ implies that the single mutations are negatively coupled (or oppose each other) in reducing specificity in the double mutant.

RESULTS

Recent findings have shown that cleavage of fibrinogen by thrombin can be selectively compromised either by targeting Glu217 near the entrance to the active site (12) or Trp215 in the aryl binding site (17). The E217A mutation abrogates a contact between the Cγ atom of Glu217 and the carbonyl O atom of Gly12 in the fibrinogen Aα chain. However, this contact is too weak to explain the significant loss (>40-fold) of specificity toward fibrinogen and fibrin (Table I). The crystal structure of thrombin inhibited with H-D-Phe-Pro-Arg-CH₂Cl, that bears a sequence identical to that of the chromogenic substrate H-D-Phe-Pro-Arg-p-nitroanilide, shows no contacts between Glu217 and substrate (19). Yet the value of $k_{cat}/K_m$ for the hydrolysis of H-D-Phe-Pro-Arg-p-nitroanilide drops over 25-fold in the E217A mutant (Table I). Cleavage of PAR1 is compromised 40-fold, but again no contact involving Glu217 is documented in the crystal structure of the thrombin-PAR1 complex (20). The effects of the E217A substitution on antithrombin III inhibition in the presence of heparin are moderate and are comparable with those on the cleavage of protein C in the absence of thrombomodulin. By contrast, the presence of thrombomodulin almost completely abolishes any perturbation of the E217A mutation, because the value of $k_{cat}/K_m$ for the hydrolysis of protein C is reduced only 30% relative to wild-type (Table I). In the case of Trp215, crystallographic evidence supports a major role in substrate recognition. This residue is absolutely conserved in thrombin from hagfish to human and is also highly conserved in the chymotrypsin family. Residue Trp215 forms a shallow cavity in the aryl binding site, together with Leu99 and Ile174 (19). The cavity shows substantial variability in shape among serine proteases (21) and is large enough to accommodate quaternary amines in some cases (22). Residue Trp215 makes an edge-to-face interaction with the benzene ring of H-D-Phe in H-D-Phe-Pro-Arg-CH₂Cl (19) and an analogous interaction with the benzene ring of Phe8 of the fibrinogen Aα chain (6). Furthermore, the P4 Leu in PAR1 makes a favorable hydrophobic interaction with Trp215 (20) and suggests that a similar contact can be made by the P4 Val residue of protein C (7). The W215A mutation produces a significant loss (>100-fold) in $k_{cat}/K_m$ for the hydrolysis of H-D-Phe-Pro-Arg-p-nitroanilide, due entirely to an increase in $K_m$ (17). The disruption of fibrinogen cleavage reaches 500-fold (Table I). By contrast, the reduction in antithrombin III inhibition and PAR1 cleavage is about 20-fold. Interestingly, the activation of protein C is substantially reduced (150-fold) in the absence of thrombomodulin, but as for the E217A mutation, addition of thrombomodulin almost abrogates the deleterious effects of the W215A mutation.

Both the E217A and W215A mutations bring about enhanced anticoagulant properties of thrombin, as documented by the relative anticoagulant potency factor (Table I). This enhancement is the result of the pronounced deleterious effect of the mutations on fibrinogen cleavage linked to a modest decrease of protein C activation in the presence of thrombo-
modulin. We therefore asked whether combining the single mutations into a double Ala mutant could further enhance this anticoagulant effect. Double mutants have been used widely in protein engineering to achieve additive effects of single mutations (23, 24). Energetic additivity of two single mutations is to be expected whenever the effects have distinct structural origins. Mutations that compromise ligand binding or substrate cleavage via a common structural mechanism are not expected to provide additive effects, because the recognition process is usually already compromised by the single substitutions. Other factors to be taken into account are the proximity of the residues involved in the mutations. Mutations of residues located far apart on the surface of the protein are usually less prone to show synergistic effects than mutations of residues close in space (18, 23, 24). Structural data support the view that Glu217 and Trp215 participate in an independent fashion to substrate recognition, but these residues are located on the same strand and are very close in both sequence and space. Hence, the resulting functional properties of the E217A/W215A double mutant, compared with those of the single Ala mutants, are of specific interest to thrombin structure-function studies but also more generally to issues of additivity of mutational effects in proteins.

The W215A/E217A double mutant has severely compromised amidolytic activity toward all substrates tested. Cleavage of H-D-Phe-Pro-Arg-p-nitroanilide is compromised over 30,000-fold, whereas cleavage of fibrinogen and fibrin are reduced 19,000- and 3,800-fold, respectively (Table I). The effects on fibrinogen and fibrin are additive (ΔGc ~ 0), whereas strong positive coupling in reducing specificity is present between the individual mutations in the hydrolysis of H-D-Phe-Pro-Arg-p-nitroanilide. The W215A/E217A double mutant releases the fibrinopeptides A and B with similar kinetics and rate constants (Fig. 1), as seen for the W215A mutant (17). This feature is not observed with the E217A mutant, and therefore the structural origin of the effect can be assigned with certainty to the interaction of Trp215 with fibrinogen. Cleavage of PAR1 by the W215A/E217A mutant is reduced 1,000-fold compared with wild-type, and the effect of the individual mutations is additive, as seen for fibrinogen and fibrin recognition.

The remarkable feature of the W215A/E217A mutant is that activation of protein C by the mutant W215A/E217A is only 7-fold slower compared with wild-type, although the enzyme concentration used in the assay is 3,000-fold higher.

![Fig. 1. Progress curves of the release of fibrinopeptides A (●) and B (○) by wild-type and the W215A/E217A mutant of thrombin.](image1)

![Fig. 2. Progress curves of the activation of protein C by wild-type (●) and the W215A/E217A mutant (○) of thrombin, in the presence of thrombomodulin.](image2)
300-fold in the absence of thrombomodulin and only 7-fold in the presence of the cofactor (Fig. 2). The presence of thrombomodulin alters the mechanism of recognition of protein C by thrombin, as demonstrated by the extraordinary increase (>1,000-fold) in specificity produced by binding of the cofactor. The molecular origin of this effect has been controversial (2, 9) until recent data proved that the enhancement of protein C cleavage by thrombin induced by thrombomodulin is due primarily to an effect of the cofactor on protein C rather than on thrombin (7, 8). The loss of specificity due to the W215A/E217A mutation is far less drastic in the presence of thrombomodulin. The individual mutations show additive effects ($\Delta G' = 0.2$ kcal/mol) in reducing specificity, whereas they act in a synergetic manner ($\Delta G' = -1.1$ kcal/mol) in the absence of cofactor. Because thrombomodulin does not change the conformation of thrombin (7, 8), the change in the coupling mode between the mutations of W215A and E217A reveals directly a thrombomodulin-induced change in the structure of the bound protein C in the transition state.

The foregoing observation is of general interest in studies of molecular recognition processes using double-mutant cycles (18, 23, 24). The precise coupling mode between individual mutations can change depending on the ligand or substrate used in the analysis, or in the presence of cofactors, because the coupling free energy between two mutations depends on the properties of the free and bound forms of the macromolecule subject to mutagenesis. Hence, conclusions derived from the analysis of the interaction of a single ligand or substrate may not reflect the true pattern of communication among individual residues subject to Ala replacement. In the case of thrombin, the coupling between mutations of Trp$^{215}$ and Glu$^{217}$ as assessed from the hydrolysis of H-o-Phe-Pro-Arg-p-nitroanilide does not coincide with that revealed by the interaction with fibrinogen or protein C in the presence of thrombomodulin and is completely reversed in the case of the hydrolysis of protein C in the absence of cofactor.

**DISCUSSION**

We have demonstrated that it is possible to rationally design thrombin derivatives with desired specificity toward a natural substrate by combining single mutations with well defined and distinct structural effects. The W215A/E217A mutant shows a value of $k_{cat}/K_m$ for the release of fibrinopeptide A from fibrinogen that is decreased 20,000-fold compared with wild-type, whereas protein C activation in the presence of thrombomodulin is compromised less than 7-fold (Table I). This gives the W215A/E217A mutant an unprecedented relative anticoagulant potency $>2,800$ that exceeds over 15-fold that of the best anticoagulant thrombin (W215A) reported to-date (17).

The W215A/E217A mutation completely reverses the relative specificity of thrombin between fibrinogen and protein C. Under physiologic conditions, the wild-type cleaves fibrinogen with a $k_{cat}/K_m$ value about 80-fold higher than that relative to the cleavage of protein C in the presence of thrombomodulin. The W215A/E217A mutant cleaves protein C in the presence of thrombomodulin with a $k_{cat}/K_m$ value about 40-fold higher than that relative to the cleavage of fibrinogen. The remarkable change in specificity translates into an anticoagulant effect that is further strengthened by a 3,000-fold reduction in the rate of inactivation by antithrombin III in the presence of heparin (Table I). This presages a drastic extension of the lifetime of the mutant in the blood compared with less potent anticoagulant thrombins already tested in vitro (12).

The W215A/E217A mutant is practically inactive toward the procoagulant substrates fibrinogen and PAR1 and should be unable to clot fibrinogen or elicit significant platelet aggregation in vitro. Under physiologic conditions where wild-type thrombin at 4 nM clots fibrinogen in about 30 s, it would take the W215A/E217A mutant at 4 nM about a week to catalyze the same reaction. By contrast, the mutant recovers almost its full activity toward protein C upon binding to thrombomodulin. If used in vitro at a physiologic concentration of 4 nM, the W215A/E217A mutant would cleave and activate protein C in the presence of thrombomodulin at a rate only 6-fold slower compared with wild-type. Increasing the concentration to 12 nM, which is certainly attainable in vivo (12), would bring the rate of activation of protein C within 50% of that of wild-type, whereas fibrinogen clotting would still require more than 2 days.

The extremely low activity toward procoagulant substrates, the insignificant rate of inhibition by antithrombin III, and the robust rate of hydrolysis of the anticoagulant substrate protein C in the presence of the physiologic cofactor thrombomodulin endow the W215A/E217A mutant with all the required properties of a potent anticoagulant thrombin (1, 14). The mutant is practically inactive toward natural substrates until it binds to thrombomodulin and therefore it can exert its anticoagulant role predominantly in the microcirculation where the thrombomodulin concentration is high and the effect is needed the most to maintain normal blood flow (2). Tests of the anticoagulant potency of this mutant in vivo should now be used to validate the exciting conclusions drawn from the studies in vitro.

**REFERENCES**