Selective Loss of Fibrinogen Clotting in a Loop-less Thrombin*

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The autolysis loop of thrombin comprises nine residues, from Glu 146 to Lys 149 e, five of which (Ala 149 a–Lys 149 e) are inserted relative to trypsin and chymotrypsin. Deletions of the insertion Ala 149 a–Lys 149 e causes no significant change in the properties of the enzyme, except for a slight enhancement of protein C activation. Deletion of the entire Glu 146–Lys 149 e loop, however, reduces fibrinogen clotting 240-fold, but decreases protein C activation only 2-fold. This loop-less mutant is de facto an exclusive activator of protein C, having lost the primary procoagulant function of thrombin. Because the autolysis loop affects fibrinogen binding, but not protein C activation, it provides a target for new drugs designed to suppress exclusively the procoagulant activity of thrombin.

Thrombin plays two major roles in blood coagulation. One is to promote clot formation upon cleavage of fibrinogen and the other is to temper the coagulation response by cleavage of protein C (1). Recent studies demonstrate that these functions can be dissociated by site-directed mutagenesis in a rather effective way, tipping the balance in favor of the anticoagulant activity of the enzyme (2, 3). An underlying property of these mutations is that they involve residues located in proximity of the Na+ binding site and tend to decrease or abolish Na+ binding thereby stabilizing the anticoagulant slow form of thrombin. An alternative way of selectively interfering with fibrinogen binding has been revealed by a thrombin modulator that inhibits fibrinogen clotting and enhances protein C activation independent of Na+ or thrombomodulin binding (4). This finding suggests that regions of the enzyme not involved directly in Na+ or thrombomodulin binding may serve as epitopes for fibrinogen, but not protein C recognition, and may provide targets for a new class of anticoagulants. One such region is identified here by site-directed mutagenesis.

MATERIALS AND METHODS

The two deletion mutants were constructed, expressed, and purified as described (3, 5). Human fibrinogen, antithrombin III, protein C, and activated protein C were purchased from Hematologic Technologies (Essex Junction, VT) and Enzyme Research (South Bend, IN). Heparin was from Sigma. Recombinant human thrombomodulin with chondroitin sulfate was from Lilly. Experimental conditions were 5 mM Tris, 145 mM NaCl, 0.1% polyethylene glycol, pH 7.4 at 37 °C. The release of fibrinopeptides was quantified by high performance liquid chromatography, and thrombomodulin binding was determined by competition with fibrinopeptide A release (6). Activation of protein C was quantified from analysis of progress curves in the presence of 5 mM CaCl2 and a saturating concentration (100 nM) of thrombomodulin (3). The inhibition of thrombin by antithrombin III in the presence of 0.5 USP units/ml of heparin was quantified from analysis of progress curves (3). Na+ binding was measured directly by fluorescence titrations using a PTI QM-1 spectrophluorometer, under experimental conditions of 275 mM thrombin, 5 mM Tris, I = 200 mM, 0.1% polyethylene glycol, pH 8.0 at 25 °C. Data were taken as triplicate measurements of intrinsic fluorescence by titrating a solution of thrombin in tetrathylenelammonium chloride with a solution of thrombin in NaCl, at the same I = 200 mM (7).

RESULTS AND DISCUSSION

A structural domain of thrombin is strategically located in between the Na+ binding site and exosite I, which provides the locale for fibrinogen (8) and thrombomodulin (9) binding (Fig. 1). This domain is the autolysis loop (10), so defined in view of its intrinsic susceptibility to proteolytic digestion, and spans nine residues, from Glu 146 to Lys 149 e. The loop distinguishes itself from the homologous region in other serine proteases like trypsin and chymotrypsin for the five-residue insertion Ala 149 a–Lys 149 e (Table I). In the crystal structure (10, 11) the autolysis loop assumes different conformations, depending on solution conditions, and seems to control the access to the active site, of which it defines the lower rim. These properties have suggested that the loop would play an important role in the remarkable selectivity of thrombin toward a limited number of physiological substrates compared with the wider specificity of trypsin (10). An unequivocal characterization of the role of this loop has, however, remained elusive.

Inspection of the sequence of the autolysis loop in thrombin from different species (12) reveals a lack of conservation for the five-residue insertion Ala 149 a–Lys 149 e (Table I). The insertion is shorter in several species, and the nature of the amino acid present at each position seems inconsequential. This suggests that only the length of the insertion should matter, since no insertion is present in trypsin and chymotrypsin. However, deletion of the Ala 149 a–Lys 149 e five-residue insertion in human thrombin produces no significant change in the functional properties of the enzyme (Table II). Fibrinogen clotting is retained and so is thrombomodulin binding and inhibition by antithrombin III. Notably, protein C activation increases 3-fold as though a shorter loop would enhance accessibility of the active site for this substrate. Binding of Na+ is also unaffected by the deletion (Fig. 2).

Unlike the insertion sequence, the sequence Glu 146–Thr 147, Trp 148 in the autolysis loop is highly conserved (Table I). Both Glu 146 and Trp 148 are present in all species, from human to hagfish. Conservation of Trp 148 is unclear, because mutation of this residue has no effect on thrombin function (5). Conservation of Glu 146, on the other hand, seems to be justified by the important ion pair formed with Arg 221a in the Na+ binding loop (3). Disruption of this ion pair in the R221aA mutant (3), or in the naturally occurring mutant E146A thrombin Salakta (13), reduces fibrinogen clotting by 6-fold with minimal effects on protein C activation due to reduced Na+ binding (3). The insertion Ala 149 a–Lys 149 e plays no role, even when the Arg 221a–Glu 146 ion pair is broken. In fact, swapping the autolysis loop of thrombin with that of trypsin (Table I) results in functional...
properties similar to those of the R221aA and E146A mutants (14). On the other hand, deletion of the Glu\textsuperscript{146}–Trp\textsuperscript{148} segment alone reduces fibrinogen clotting 20-fold and protein C activation 16-fold (15).

Given these previous findings and the properties of the deletion mutant ΔAla\textsuperscript{149a}–Lys\textsuperscript{149e} (Table II), we expected from simple additive effects that deletion of all nine residues Glu\textsuperscript{146}–Lys\textsuperscript{149e} would compromise both fibrinogen and protein C binding to similar extent. On the other hand, the ΔGlu\textsuperscript{146}–Lys\textsuperscript{149e} mutant has practically lost its clotting activity because of the remarkable reduction in the release of fibrinopeptides A and B by 240- and 520-fold, respectively, whereas its ability to activate protein C is reduced only 2-fold (Table II). Binding of thrombomodulin is reduced 6-fold, inhibition by antithrombin III is reduced 20-fold, and Na\textsuperscript{+} binding is reduced over 10-fold (Fig. 2).

Some of the properties of the loop-less mutant can be explained in terms of a perturbation of the slow ↔ fast equilibrium. Thrombomodulin binds to the loop-less mutant with the same affinity as the slow form of thrombin, consistent with this mutant being essentially in the slow form under physiological conditions. Hence, the autolysis loop plays no role in thrombomodulin binding. A somewhat similar explanation can be invoked for antithrombin III and protein C. The loss in specificity experienced by these molecules in binding to the mutant compared with the slow form echoes that observed for the R221aA mutant (3) and likely originates from disruption of the Arg\textsuperscript{221a}–Glu\textsuperscript{146} ion pair. The broken ion pair in R221aA also reduces the Na\textsuperscript{+} affinity as seen for the loop-less mutant. The effect on fibrinogen, on the other hand, cannot be reconciled entirely with a perturbation of the slow ↔ fast equilibrium in favor of the slow form, that would reduce specificity 10-fold relative to wild-type (Table II). Most of the effect must originate from direct perturbation of fibrinogen binding due to the loss of critical interactions in the loop-less mutant. Because none of the residues deleted in the loop-less mutant makes contact with fibrinopeptide A (8), other portions of the fibrinogen molecule must interact with the autolysis loop. The possibility that the conformation of other regions of thrombin interacting exclusively with fibrinogen is altered in the loop-less mutant cannot be ruled out, although the modest effect seen for protein C, antithrombin III and thrombomodulin binding excludes a drastic perturbation of thrombin structure. The crystal structure of the loop-less mutant will help clarify this point.

Independent of whether the autolysis loop participates directly in fibrinogen binding or influences other regions of the enzyme, these results demonstrate that deletion of the loop selectively suppresses fibrinogen clotting. The 240-fold reduction in the release of fibrinopeptide A with a concomitant 2-fold loss of protein C activation implies that use of this mutant in vivo at twice the concentration as wild-type thrombin would generate the same amount of activated protein C, but would clot blood in 8 h instead of 4 min (16). Given the limited life-span of thrombin in the blood (10–15 min) due to scavenging by antithrombin III (16), the loop-less mutant is de facto an exclusive activator of protein C. Its properties are extraordi-

![Fig. 1. MOLSCRIPT plot of the B chain of human thrombin (11) showing the spatial location of the catalytic triad relative to important structural domains, as noted. The bound Na\textsuperscript{+} is shown by a circle. The autolysis loop comprising the nine residues Glu\textsuperscript{146}–Lys\textsuperscript{149e} is located in between the Na\textsuperscript{+} binding site and exosite I. Deletion of this loop results in the selective loss of fibrinogen clotting.](image)

**TABLE I**

| Sequence of the autolysis loop in thrombin from different species (* denotes a deletion) |
|---------------------------------------------|
| 146 | 147 | 148 | 149 | 168a | 168b | 169a | 169b | 190a | 190b | 191 | 192 | 193 | 194 | 195 | 196 | 197 | 198 | 199 | 200 | 201 |
|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| human | R | T | W | T | N | V | G | K | | | | | | | | | | | | | |
| mouse | R | T | W | T | N | I | E | | | | | | | | | | | | | | | |
| rat | R | T | W | T | N | I | E | | | | | | | | | | | | | | | |
| rabbit | R | M | W | T | N | V | M | N | | | | | | | | | | | | | |
| bovine | R | T | W | T | S | V | A | E | | | | | | | | | | | | | |
| chicken | R | T | W | T | S | V | A | E | | | | | | | | | | | | | |
| gecko | R | T | W | G | S | S | T | P | | | | | | | | | | | | | |
| newt | R | T | W | S | G | S | G | Q | | | | | | | | | | | | | |
| rainbow trout | R | T | W | S | S | P | K | K | | | | | | | | | | | | | |
| sturgeon | R | T | W | S | S | S | | | | | | | | | | | | | | | | |
| hagfish | B | M | W | S | L | S | S | E | | | | | | | | | | | | | |
| chymotrypsin | Y | T | N | A | * | * | * | * | | | | | | | | | | | | | |
| trypsin | S | S | G | T | * | * | * | * | | | | | | | | | | | | | |

**TABLE II**

| Specificity of wild-type and deletion mutants of the autolysis loop under physiological conditions |
|-----------------------------|
|                          | Slow form\(^\text{a}\) | Fast form\(^\text{a}\) | Wild-type\(^\text{a}\) | ΔAla\textsuperscript{149a}–Lys\textsuperscript{149e} | ΔGlu\textsuperscript{146}–Lys\textsuperscript{149e} |
| Fibrinopeptide A release, \(k\text{cat}/K\text{m} (\text{M}^{-1} \text{s}^{-1})\) | 1.5 ± 0.1 | 35 ± 4 | 17 ± 1 | 19 ± 1 | 0.071 ± 0.003 |
| Fibrinopeptide B release, \(k\text{cat}/K\text{m} (\text{M}^{-1} \text{s}^{-1})\) | 0.73 ± 0.04 | 17 ± 1 | 9.4 ± 0.5 | 7.0 ± 0.4 | 0.018 ± 0.001 |
| Protein C activation, \(k\text{cat}/K\text{m} (\text{M}^{-1} \text{s}^{-1})\) | 0.32 ± 0.01 | 0.21 ± 0.01 | 0.22 ± 0.01 | 0.58 ± 0.06 | 0.11 ± 0.01 |
| Thrombomodulin binding, \(K\text{d} (\text{M})\) \(\text{cat/}\) | 5.3 ± 0.7 | 0.60 ± 0.02 | 0.99 ± 0.04 | 1.2 ± 0.1 | 6.4 ± 0.1 |
| Antithrombin III inhibition, \(k\text{cat} (\text{M}^{-1} \text{s}^{-1})\) \(\text{cat/}\) | 4.0 ± 0.2 | 20 ± 2 | 13 ± 1 | 21 ± 1 | 0.55 ± 0.01 |

\(^{a}\) Data from (3).

\(^{b}\) Relative anticoagulant potency (3) of a mutant, \((a_{\text{mut}}/c_{\text{mut}})/(a_{\text{wt}}/c_{\text{wt}})\), where \(a\) and \(c\) are the rates for cleavage of protein C and release of fibrinopeptide A. The suffix wt refers to wild-type under physiological conditions.
narily similar to those of the E217K mutant recently engineered for optimal anticoagulant activity in vivo (17) and represent a substantial gain in anticoagulant potency compared with other single-site substitutions (2, 3).

The most important implication of these results is that the autolysis loop represents an ideal target for molecules aimed at suppressing exclusively the procoagulant activity of the enzyme. An active-site inhibitor of thrombin would shut down both procoagulant and anticoagulant activities, whereas a drug that selectively suppresses fibrinogen binding would have an enhanced anticoagulant potency in view of the lack of inhibition of the protein C-dependent anticoagulant pathway. The pursuit of such thrombin modulators, fostered by the discovery of the allosteric nature of this enzyme (18), is quite realistic (4) and should be greatly facilitated by the identification of suitable molecular targets like the autolysis loop. Targeting this loop, that is more conspicuous in thrombin than trypsin, should also reduce the cross-reactivity of the modulator with the digestive protease that so often compromises the biological potency of active-site inhibitors of thrombin designed for oral availability.

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