Thrombomodulin Enhances the Reactivity of Thrombin with Protein C Inhibitor by Providing Both a Binding Site for the Serpin and Allosterically Modulating the Activity of Thrombin*

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Thrombomodulin (TM), or its epidermal growth factor-like domains 456 (TM456), enhances the catalytic efficiency of thrombin toward both protein C and protein C inhibitor (PCI) by 2–3 orders of magnitude. Structural and mutagenesis data have indicated that the interaction of basic residues of the heparin-binding exosite of protein C with the acidic residues of TM4 is partially responsible for the efficient activation of the substrate by the thrombin-TM456 complex. Similar to protein C, PCI has a basic exosite (H-helix) that constitutes the heparin-binding site of the serpin. To determine whether TM accelerates the reactivity of thrombin with PCI by providing a binding site for the H-helix of the serpin, an antithrombin (AT) mutant was constructed in which the H-helix of the serpin replaced with the same region of PCI (AT-PCIH₄-helix). Unlike PCI, the H-helix of AT is negatively charged. It was discovered that TM456 slightly (2-fold) impaired the reactivity of AT with thrombin; however, it enhanced the reactivity of AT-PCIH₄-helix with the protease by an order of magnitude. Further studies revealed that the substitution of Arg³⁶ of thrombin with an Ala also resulted in an order of magnitude enhancement in reactivity of the protease with both PCI and AT-PCIH₄-helix independent of TM. We conclude that TM enhances the reactivity of PCI with thrombin by providing both a binding site for the serpin and a conformational modulation of the extended binding pocket of thrombin.

Protein C inhibitor (PCI) is an inhibitor of the serpin superfamily that can react with most coagulation proteases in plasma (1–4). It has been named PCI because it was initially thought that its main target in plasma is activated protein C. However, several years ago we demonstrated that the main plasma target protease for PCI is the thrombin-thrombomodulin (TM) complex (5). This was evidenced by the observation that TM enhanced the reactivity of thrombin with PCI by two orders of magnitude. The cofactor effect of TM in promoting the reactivity of PCI with thrombin was because of protein-protein interactions, because the epidermal growth factor-like domains 456 (TM456) of the cofactor, which is devoid of the heparin-like chondroitin sulfate moiety, accelerated the reaction to a similar extent (5). TM456 is the minimal functional fragment of the cofactor that is capable of switching the specificity of thrombin from a procoagulant to an anticoagulant enzyme (6, 7). TM456 binds to exosite-1 of thrombin and competitively inhibits the binding of procoagulant ligands such as fibrinogen, PAR1, and procofactors V and VIII to this site of thrombin (8–13). Moreover, TM456 dramatically improves the catalytic efficiency of thrombin toward protein C, thereby initiating the anticoagulant pathway by rapidly activating the substrate to activated protein C (6). Activated protein C down-regulates the coagulation cascade by degrading both factors Va and VIIIa by limited proteolysis (14). Paradoxically, TM also improves the catalytic efficiency of thrombin toward the thrombin-activable fibrinolysis inhibitor (15). Activated thrombin-activable fibrinolysis inhibitor is a carboxypeptidase B-like enzyme that can down-regulate fibrinolysis by cleaving carboxy-terminal basic residues of fibrin. Thus, plasma PCI may play an important role in the regulation of both coagulation and fibrinolytic pathways (16).

The mechanism by which TM modulates the interaction of thrombin with different target plasma proteins is not known. In the case of protein C activation, molecular modeling based on the structure of the thrombin-TM456 complex indicated that the binding of the TM5 domain of the cofactor to exosite-1 of thrombin orients the TM4 domain in such a way that a negatively charged region in this domain contacts a basic exosite of protein C in the ternary activation complex (17). The basic residues of this exosite are known to constitute a heparin-binding exosite in both protein C and activated protein C (18, 19). It has been postulated that electrostatic interactions between the oppositely charged residues of this exosite of protein C and the TM4 domain of the cofactor in the activation complex facilitate a proper docking of the substrate into the catalytic pocket of thrombin (17). In support of this hypothesis, we recently used a compensatory charge reversal mutagenesis approach and demonstrated that the TM4 domain provides a binding site for interaction with the heparin-binding exosite of the substrate in the ternary thrombin-TM456-protein C complex (20).

PCI is a heparin-binding serpin the structure of which, similar to other serpins, is comprised of three β-sheets and nine α-helices (21, 22). However, in contrast to other heparin-binding serpins which interact with the polysaccharide via their basic residues of the D-helix, in the case of PCI the basic H-helix constitutes the heparin-binding exosite of the serpin.
Inhibitory properties of the AT mutant toward thrombin in expressed in HEK293 cells and purified to homogeneity by an immunoaffinity column, washed with high salt, and eluted with calibrated heparin as monitored from chromatography with a gradient elution from 0.1 to 2.0M NaCl in 20 mM Tris-HCl, pH 7.5, as described (24). Concentrations of the AT mutant with thrombin was significantly diminished if certain TM4 charge reversal mutants of TM456 replaced the wild type cofactor in the inhibition reactions. Further studies revealed that the reactivity of both PCI and AT-PCI^H-helix with the Arg35 → Ala mutant of thrombin was also improved by an order of magnitude independent of TM. These results suggest that TM accelerates the PCI inhibition of thrombin by providing both a binding site for the H-helix of the serpin and an allosteric modulation of the 39-loop of thrombin.

**EXPERIMENTAL PROCEDURES**

**Construction and Expression of the Recombinant Proteins**—The expression of wild type AT by the RSV-PL4 mammalian expression/purification vector system in HEK293 cells has been described (24). The chimeric AT-PCI^H-helix mutant in which the sequence of the H-helix of PCI was replaced with the corresponding sequence of PCI (269Arg-Lys-Trp-Leu-Lys-Met-Asn) was constructed by PCR mutagenesis methods as described (24). Another AT mutant in which the acidic residues Asp309, Glu310, Glu312, and Glu313 of the serpin were replaced with the isosteric residues Asn and Gln (AT-Neu^H-helix) was also constructed in the same vector system. Following confirmation of the accuracy at 280 nm, assuming a molecular mass of 36.6 kDa and absorption coefficient (ε280) of 17.1 (25). The concentrations of active enzymes were determined as described above.

### Determination of Inhibition Stoichiometry (SI)

The SI values for the inhibition of thrombin by the serpins were determined by titration of 20 nM thrombin, alone or in complex with 250 nM TM456 with increasing concentrations of each serpin corresponding to serpin/thrombin molar ratios of 0.2. The residual amidolytic activity of thrombin was monitored at 5 to 10 h at room temperature by the chromogenic substrate Spectrozyme TH as described above. After completion of the inhibition reactions, the residual amidolytic activity was plotted versus the residual activity of the enzyme, and the SI values were determined from the x-intercept of the linear regression fit of the inhibition data as described (25).

### RESULTS

**Expression and Purification of Recombinant Proteins**—Wild type and the H-helix mutants of AT were expressed in HEK293 cells and purified to homogeneity by a combination of immunoaffinity and HiTrap-heparin chromatography as described (24). Following chromatography on the heparin column, the purified proteins were reapplied to the Ca2+-dependent HPC4 immunoaffinity column, washed with high salt, and eluted with EDTA as described (24). The second immunoaffinity chromatography was performed to eliminate possible traces of heparin contamination. SDS-PAGE analysis indicated that recombinant proteins were purified to homogeneity (Fig. 1). Both the wild type and H-helix mutant serpins migrated at the expected molecular mass of ~60 kDa. The amino acid residues forming the H-helices of PCI and AT mutants are presented in Fig. 1. The expression and purification of TM456 derivatives in which the charges of basic (Arg) and acidic residues of TM4 (Asp, Glu) were reversed by their substitutions with a Glu or Arg/Lys, respectively, were described previously (20). The R35A and E39Q thrombin mutants were prepared as described (27, 28). Previous characterization of both mutants of thrombin has indicated that mutations of either Arg27 or Glu28 (27) reversibly affects the catalytic pocket of thrombin.

The purity of all recombinant proteins was checked by SDS-PAGE. Similar to wild type, the H-helix mutants of AT...
formed high molecular weight complexes with both thrombin and the thrombin-TM456 complex as determined by SDS-PAGE (data not shown).

Inhibition of Thrombin in the Absence and Presence of TM456—The concentration dependence of the observed pseudo first-order rate constants ($k_{\text{obs}}$) for the wild type AT inhibition of thrombin and the thrombin-TM456 complex is presented in Fig. 2A. TM456 slightly impaired the reactivity of AT with thrombin. However, the reactivity of the AT-PCIH-helix mutant with thrombin was markedly improved both in the absence and presence of TM456. As shown in Fig. 2B, AT-PCIH-helix inhibited thrombin ~7-fold better than wild type AT in the absence of the cofactor (Table I). TM456 further improved the reactivity of AT-PCIH-helix with thrombin by an order of magnitude (Fig. 2A). Thus, relative to the second-order rate constant ($k_2$) for the thrombin-TM456-AT reaction ($6.3 \times 10^5$ M$^{-1}$ s$^{-1}$), the corresponding value for the reaction with AT-PCIH-helix (5.8 $\times$ 10$^5$ M$^{-1}$ s$^{-1}$) was elevated 92-fold (Table I). This value is only ~2-fold lower than the corresponding value for the PCI inhibition of the thrombin-TM456 complex. On the other hand, the reactivity of AT-NeuH-helix with thrombin was improved 2.5-fold, and TM456 accelerated the reaction less than 2-fold (Fig. 2C, Table I). These results suggest that electrostatic interactions between TM and basic residues of the H-helix may account for the improvement in the reactivity of AT-PCIH-helix with thrombin in the ternary complex. It should be noted that both AT and PCI inhibited wild type and mutant thrombins with a similar SI value of ~1.0–1.1 in both the absence and presence of TM456. However, the SI value for the AT-PCIH-helix with both wild type and mutant thrombins was elevated to ~1.4–1.5 in both the absence and presence of TM456. The slight increase in the rate of the substrate pathway of the mutant serpin with thrombin was not included in the calculation of the overall rate constants presented in Table I.

Based on the structural data (17), we recently demonstrated that the interaction of acidic residues of TM4 with a basic exosite of protein C contributes to the cofactor effect of TM in the rapid activation of protein C by the thrombin-TM456 complex (20). To determine whether the interaction of same acidic residues of TM4 are also responsible for the cofactor-mediated
TM Enhances the Reactivity of Thrombin with PCI

The second-order rate constants for the inhibition of the wild type, R35A, and E39Q thrombins (1 nM) by the wild type and mutant serpins in both the absence and presence of TM456 (250 nM) were determined by a discontinuous assay method as described under “Experimental Procedures.” The ratio (+TM/–TM) represents the fold TM-mediated rate enhancements in the reactivity of thrombins with serpins. All values are the averages of at least three measurements ± S.D. values.

| Second-order inhibition rate constants (in M⁻¹ s⁻¹) for the reaction of wild type, R35A, and E39Q thrombins with the wild type and mutant serpins in the absence and presence of TM456 |
|---|
| **No cofactor** | **+TM** | **+TM/–TM** |
| **Thrombin** | | |
| AT | (8.4 ± 0.9) x 10⁴ | (6.3 ± 0.6) x 10⁴ | 0.75 |
| PCI | (3.3 ± 0.3) x 10⁴ | (1.3 ± 0.2) x 10⁴ | 39.4 |
| AT-PCIH-helix | (6.1 ± 0.7) x 10⁴ | (5.8 ± 0.3) x 10⁴ | 9.5 |
| AT-NeuH-helix | (2.0 ± 0.1) x 10⁴ | (3.5 ± 0.1) x 10⁴ | 1.75 |
| **R35A thrombin** | | |
| AT | (1.7 ± 0.4) x 10⁴ | (1.1 ± 0.1) x 10⁴ | 0.65 |
| PCI | (4.2 ± 0.1) x 10⁴ | (2.6 ± 0.3) x 10⁴ | 6.2 |
| AT-PCIH-helix | (2.7 ± 0.3) x 10⁴ | (2.0 ± 0.1) x 10⁴ | 7.4 |
| **E39Q thrombin** | | |
| AT | (1.4 ± 0.1) x 10⁴ | (1.5 ± 0.1) x 10⁴ | 1.1 |
| PCI | (8.1 ± 0.8) x 10⁴ | (1.3 ± 0.1) x 10⁴ | 16.0 |
| AT-PCIH-helix | (7.1 ± 0.5) x 10⁴ | (4.7 ± 0.2) x 10⁴ | 6.6 |

FIG. 3. Loss of cofactor-mediated enhancements in the reactivity of AT-PCIH-helix and PCI with thrombin in complex with the charge reversal mutants of TM4. A, the k₂ values for the AT-PCIH-helix inhibition of thrombin (1 nM) in complex with TM456 derivatives (500 nM) were determined as described under “Experimental Procedures.” B, the same as A except that the TM-mediated enhancements are shown for the PCI inhibition of thrombin.

(31, 32). Because PCI has an Arg at the P₃ position of the reactive site loop, we examined the reactivity of Arg²⁵ → Ala (R35A) and Glu¹⁹ → Gln (E39Q) mutants of thrombin with the mutant and wild type serpins in the absence and presence of TM456. In the presence of TM456, first the optimal concentrations of TM456 in the inactivation reactions were determined for mutant thrombins. TM456 accelerated the inactivation of both wild type and E39Q thrombins by both AT-PCIH-helix and PCI with an identical apparent dissociation constant of ~5 nM (Fig. 4, shown for AT-PCIH-helix only). This value, however, was increased to ~15 nM for R35A thrombin. This result is consistent with the literature (27). It was interesting to discover that the reactivity of the R35A thrombin with AT, PCI, and AT-PCIH-helix in the absence of TM456 was improved ~2-, 13-, and 4-fold, respectively. Similar to the reaction of thrombin with wild type AT, TM456 (250 nM) slightly impaired the reactivity of R35A thrombin with the serpin (Table I). However, unlike differences in the rate-accelerating effect of TM456 on the reaction of thrombin with PCI and AT-PCIH-helix, the cofactor promoted the rate of R35A thrombin with both serpins to a similar extent (6–7-fold, Table I). The reactivity of E39Q thrombin with both AT and PCI was also improved ~2-fold in the absence but not in the presence of TM456 (Table I). However, no significant change in the reactivity of E39Q thrombin with AT-PCIH-helix was observed in either the absence or presence of the cofactor (Table I).

**DISCUSSION**

The results of this study suggest that differences in the sequences of H-helices between PCI and AT (Fig. 1) are primarily responsible for their differential reactivity with thrombin in both the absence and presence of TM. This is evidenced by the observation that PCI and AT-PCIH-helix inhibited thrombin at approximately the same rate in both the absence and presence of TM456 (Table I). Thus, the interaction of basic residues of the H-helix of PCI with the acidic residues of TM4 in the ternary thrombin-TM-PCI complex appears to be responsible for the improved reactivity of the protease with the serpin. However, unlike an ~40-fold TM-mediated enhancement in the
reactivity of PCI with thrombin, there was an ~10-fold enhancement in the reactivity of AT-PCIH-helix with thrombin in the presence of TM456 (Table I). This may partly be because of the observation that the reactivity of the AT-PCIH-helix mutant with thrombin was significantly improved independent of TM. Two questions must be addressed here. First, what is the molecular basis for the enhanced reactivity of AT-PCIH-helix with thrombin in the absence of the cofactor? Second, if TM functions by providing a binding site for the serpin in the complex, why did it not then enhance the reactivity of AT-PCIH-helix with thrombin to a similar extent? At least two possibilities may account for the TM-independent improvement in the reactivity of thrombin with AT-PCIH-helix. First, because the reactive site loop of AT is trapped in a partially loop-inserted inactive conformation (33), one possibility is that the substitution of the H-helix of AT with the H-helix of PCI has resulted in the activation of the mutant serpin, thereby improving its reactivity with thrombin. To test this possibility, $k_2$ values for the AT-PCIH-helix inhibition of factor Xa were determined in both the absence and presence of a distinct pentasaccharide fragment of heparin which is known to bind and activate AT, thereby improving the reactivity of the serpin with factor Xa 200–300-fold (34). It was found that both wild type AT and AT-PCIH-helix inhibited factor Xa with identical $k_2$ values in both the absence (2.7 x 10^3 m^-1 s^-1) and presence of the pentasaccharide (8.2 x 10^2 m^-1 s^-1), which suggested that the loop swapping did not lead to the activation of the serpin. The second possibility is that the acidic residues in the H-helix of AT are involved in repulsive interactions with an unknown site of thrombin and that the neutralization or the reversal of their charges eliminates the inhibitory interactions between the two proteins. The validity of this hypothesis cannot be determined at the present time.

At least two possibilities may account for differences in the extent of the cofactor effect of TM in accelerating the protease inhibition reactions by two serpins. First, it is possible that the binding of TM to the H-helix of PCI is also associated with a conformational change in the reactive site loop of the serpin that is not realized in the reactive site loop of the AT-PCIH-helix mutant. A noticeable difference between the P3–P3 residues of the reactive site loop of PCI (Thr-Phe-Arg-Ser-Ala-Arg) and AT (Ala-Gly-Arg-Ser-Leu-Asn) is the occurrence of a favorable Arg at the P3 position of PCI. It is known that Glu39 of thrombin is critical for determining the P3-binding specificity of thrombin. Nevertheless, previous PCI reactive site loop mutagenesis studies have indicated that the substitution of the P3'-Arg of PCI with an Ala or an Asp minimally alters the reactivity of mutant serpins with thrombin (23). Subsequent molecular modeling of the thrombin-PCI interaction has revealed that the P3'-Arg may not interact with Glu39 of thrombin, because it is directed toward the body of PCI (23). Thus, one possibility is that the interaction of the H-helix with TM in PCI alters the conformation of the P3'-Arg of the serpin, thereby improving its reactivity with thrombin. However, a similar wild type-reactivity of E39Q thrombin with PCI in the presence of TM456 (1.3 x 10^8 m^-1 s^-1) did not support this hypothesis. It should be noted that the P2 residues also play an important role in determining the serpin specificity of thrombin and other coagulation proteases (35, 36). Thrombin prefers a Pro at the P2 position, and thus the P2 residues of both serpins are non-optimal for interaction with thrombin. It appears, therefore, that the apparent differences in the sequence of the reactive site loops of two serpins do not significantly contribute to their differential reactivity with thrombin. This may not be surprising because an important reactive site loop structural feature that is critical for the reaction with thrombin has been conserved in both serpins. The reactive site loop of both serpins has an insertion of three residues (Arg-Val-Thr in AT and Ser-Gln-Arg in PCI (21)). We previously showed that whereas a longer reactive site loop is inhibitory for the AT interaction with factor Xa, it is required for the optimal reaction of the serpin with thrombin (37). Thus, differences in the H-helices of two serpins appear to be primarily responsible for their differential reactivity with thrombin in both the absence and presence of TM.

The alternative possibility is that TM induces conformational changes in the substrate-binding pocket of thrombin, thereby differentially influencing the reactivity of the protease with the two serpins. One of the surface loops in thrombin that may be the target for an allosteric modulation by TM is Arg35 on the 35–39-loop. Structural and mutagenesis data suggest that the guanidyl group of Arg35 contacts Glu39 and points toward the active site pocket of thrombin (31, 32), which can potentially impede the access of the active site pocket of thrombin by PCI and other target macromolecules. To test the possibility that the TM modulation of the 35–39-loop of thrombin accounts for the differences in the cofactor activity of TM, the reactivity of PCI and AT-PCIH-helix was examined with R35A thrombin in both the absence and presence of TM456. Interestingly, it was discovered that the reactivity of PCI with R35A thrombin was improved ~13-fold independent of TM (Table I). The reactivity of AT-PCIH-helix with the mutant thrombin was improved ~4-fold under the same conditions. Moreover, instead of a 2-fold higher reactivity for AT-PCIH-helix with thrombin, there was a 2-fold higher reactivity for PCI with R35A thrombin in the absence of TM. Thus, R35A thrombin reacted with both PCI and AT-PCIH-helix with similar $k_2$ values in the presence of TM (Table I). These results suggest that TM allosterically modulates the 35–39-loop of thrombin. Thus, the difference in the extent of the cofactor effect of TM with the two serpins is because of the difference in the extent of the cofactor effect of TM on thrombin through an allosteric mechanism.

In summary, in this study we demonstrated that the H-helix of PCI interacts with TM4 of the thrombin-TM456 complex. This interaction appears to account for an order of magnitude improvement in the reactivity of the serpin with the anticoagulant thrombin. A TM-mediated conformational change in the 35–39-loop of thrombin appears to lead to another order of magnitude enhancement in the reactivity of PCI with thrombin. Thus, TM accelerates the PCI inhibition of thrombin by both modulation of the extended binding pocket of thrombin and by a “substrate presentation” mechanism. Both mechanisms appear to make equal contributions to the acceleration of thrombin inhibition by the serpin. TM also enhances protein C activation by thrombin by similar mechanisms. Thus, these may be common mechanisms by which cofactors modulate the inhibitor and substrate specificity of coagulation proteases.

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