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Protein C Inhibitor Is a Potent Inhibitor of the Thrombin-Thrombomodulin Complex*

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Protein C inhibitor (PCI), a plasma serine protease inhibitor, inhibits several proteases including the anticoagulant enzyme, activated protein C (APC), and the coagulation enzymes, thrombin and factor Xa. Previous studies have shown that thrombin and APC are inhibited at similar rates by PCI and that heparin accelerates PCI inhibition of both enzymes more than 20-fold. We now demonstrate that the thrombin-binding proteoglycan, rabbit thrombomodulin, accelerates inhibition of thrombin by PCI ≈140-fold (k_2 = 2.4 × 10^6 in the presence of TM compared to 1.7 × 10^4 M^{-1} s^{-1} in the absence of TM). Most of this effect is mediated by protein-protein interactions since the active fragment of TM composed of epidermal growth factor-like domains 4–6 (TM 4–6) accelerates inhibition by PCI ≈59-fold (k_2 = 1.0 × 10^6 M^{-1} s^{-1}). The mechanism by which TM alters reactivity with PCI appears to reside in part in an alteration of the S2 specificity pocket. Replacing Phe^{353} with Pro at the P2 position in the reactive loop of PCI yields a mutant that inhibits thrombin better in the absence of TM (k_2 = 6.3 × 10^5 M^{-1} s^{-1}), but TM 4–6 enhances inhibition by this mutant ≈9-fold (k_2 = 5.8 × 10^6 M^{-1} s^{-1}) indicating that TM alleviates the inhibitory effect of the less favored Phe residue. These results indicate that PCI is a potent inhibitor of the protein C anticoagulant pathway at the levels of both zymogen activation and enzyme inhibition.

Protein C inhibitor is a heparin-binding plasma serine protease inhibitor (serpin)[1] (1–4). *In vivo* and in plasma, a significant percentage of activated protein C (APC) is inhibited by PCI with various studies reporting 10–50% of the APC in complex with PCI (5–9). The other major inhibitor of APC is α_1-antitrypsin. Heparin accelerates inhibition of APC by PCI, but not by α_1-antitrypsin.

Originally identified as an inhibitor of activated protein C, it is now apparent that this inhibitor has a broad specificity, inhibiting several of the blood coagulation enzymes including thrombin and factor Xa. In fact, PCI inhibits thrombin better than APC both in the presence and absence of heparin (4, 10). This paradoxical effect of inhibition of both coagulation and anticoagulation proteins raises questions about the physiological role of PCI.

Thrombin serves a dual role in coagulation. It clots fibrinogen, activates platelets, and feeds back to promote coagulation by activating cofactors (11). Alternatively, thrombin can bind to thrombomodulin (TM), and this complex accelerates protein C activation giving rise to the anticoagulant serine protease, APC (12). APC then prevents further thrombin formation by inactivating factors Va and VIIIa, two cofactors required for thrombin generation (12, 13). *In vivo*, thrombin inhibition is usually believed to be due primarily to antithrombin, another heparin-binding serpin. This inhibition can be catalyzed by vascular proteoglycans or by thrombin binding to TM (14, 15). TM is a proteoglycan containing a covalently associated chondroitin sulfate moiety. Only forms of TM that contain the chondroitin sulfate enhance inhibition of thrombin by antithrombin (16–18). The chondroitin sulfate moiety is not, however, required for protein C activation, a process that appears to involve conformational changes in the extended binding pocket of thrombin (19, 20). The influence of TM on thrombin inhibition by PCI has not been examined fully.

In the present study, we demonstrate that TM potently accelerates inhibition of thrombin by PCI, a process that depends primarily on protein-protein interactions between thrombin and TM. These studies provide new insights into the possible physiological functions of PCI.

EXPERIMENTAL PROCEDURES

Proteins and Reagents—Recombinant human thrombin was prepared as described previously (21). The concentration of purified recombinant thrombin was determined by active site titration with *p*-nitrophenyl *p′*-guanidinobenzoate and agreed with the calculated concentration based on absorbance at 280 nm (21). Recombinant human PCI and Phe^{353} → Pro mutant of PCI were expressed and purified from a baculovirus expression system as described previously (3, 22). The concentration of recombinant PCI was calculated based on thrombin active site titration and immunoassay as described (3). Recombinant human TM 4–6 fragment (23) and rabbit lung TM (24) were isolated by the cited methods. All proteins were homogeneous by acrylamide gel electrophoresis in sodium dodecyl sulfate. Polybrene was purchased from Sigma. Spectrozyme TH was purchased from American Diagnostica, Greenwich, CT, and S2266 was purchased from Kabi Pharmacia/Chromogenix, Franklin, OH.

Thrombin Inhibition with PCI—The rate of inhibition of human thrombin was measured under pseudo-first order rate conditions by

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[1] The abbreviations used are: serpin, serine protease inhibitor; PCI, protein C inhibitor; APC, activated protein C; TM, thrombomodulin; TM 4–6, recombinant epidermal growth factor-like repeats 4–6 of TM spanning residues Val^{348} to Gly^{465} prepared by the recombinant DNA methods; PCI F353P, protein C inhibitor mutant in which Phe at position 353 is converted to Pro; SPTH, Spectrozyme TH.

both discontinuous and continuous assay methods. In the discontinuous assay method, both the inhibition reaction and the determination of residual thrombin activity were done in 96-well plates at room temperature in 20 mM Tris-HCl (pH 7.5), 0.1 M NaCl (TBS) containing 2 mg/ml bovine serum albumin, as described (3, 4). Thrombin (0.5 nM) was incubated with at least a 10-fold excess of PCI (10 nM) at room temperature in the presence and absence of saturating concentrations of TM (50 nM) or TM 4–6 (100 nM). For assays in the absence of TM, 0.1 mg/ml Polybrene was also included in the buffer. Polybrene was added to ensure that trace heparin contamination of PCI would not influence the inhibition rate. However, the rates of PCI inhibition of thrombin in the presence or absence of Polybrene were similar indicating that PCI preparations were essentially free of heparin. After a period of time (30 s to 30 min depending on the reaction rates), Spectrozyme TH (SPTH) in TBS buffer containing 1 mg/ml Polybrene (to block the chondroitin sulfate moiety of TM) was added to give a final concentration of 0.2 mM. Following color development, the absorbance at 405 nm was measured with a V_{max} Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA). Rate constants were calculated using the equation:

$$k_2 = (-\ln a)/t[\mathrm{I}] \qquad \text{(Eq. 1)}$$

where a is residual proteinase activity, t is time, and [I] is the PCI concentration (3, 4). All experiments were performed in triplicate wells, and all experiments contained control wells in which the assay buffer had replaced PCI. In all experiments it was ensured that less than 10% chromogenic substrate was utilized and all inhibition assays were performed by time course analysis to obtain at least 50% enzyme inhibition for calculation of inhibition rates.

As mentioned above, the PCI concentration in this discontinuous assay method was 10 nM. In the presence of TM and higher concentrations of PCI (*i.e.* 30 nM), the rate of inactivation was fast, and, in 15 s, more than 90% of thrombin activity was inhibited. To demonstrate the concentration dependence of PCI inhibition in the presence of TM, the alternative method of continuous inhibition assay was employed. In this method, the inhibition reaction was carried out in the presence of a competing chromogenic substrate as described (20, 25). In this case, 50 μl of 1 nM human thrombin (0.5 nM final) in complex with saturating concentrations of TM (50 nM final) or TM 4–6 (100 nM final) were added to wells of a 96-well plate that contained 50 μl of PCI at final concentrations ranging from 3.1 to 100 nM and SPTH (0.2 mM final), and the absorbance at 405 nm was measured at 20-s time intervals immediately after thrombin addition. S2266 (0.5 mM) with a lower affinity for thrombin was employed to monitor thrombin inhibition by PCI in the absence of TM. The PCI concentration had to be increased to 250 nM to obtain reasonable rates of inhibition under these conditions. The K_m values that were determined and used in Equation 3 were 5.6 μM for SPTH with thrombin, 6.0 μM for the rabbit TM-thrombin complex, and 5.8 μM for the TM 4–6-thrombin complex. The K_m of thrombin for S2266 was 236 μM.

The apparent pseudo-first order rate constant of inhibition was estimated by fitting the absorbance at 405 nm *versus* time into the following equation:

$$Ab = A_0/k_{app} \times (1 - e^{-k_{app} \times t}) + Ab_0 \qquad \text{(Eq. 2)}$$

where t is the time of the inhibition, Ab is the absorbance at 405 nm at time t, Ab_0 is the absorbance at 405 nm at time 0, A_0 is the thrombin activity at time 0, and k_{app} is the apparent pseudo-first order rate constant of inhibition. To correct for the presence of chromogenic substrate, the pseudo-first order rate constant of inhibition k' was given by:

$$k' = k_{app} \times (1 + [\mathrm{S}]/K_m) \qquad \text{(Eq. 3)}$$

where [S] is the concentration of the chromogenic substrate, SPTH or S2266, and K_m is the Michaelis-Menten constant of thrombin for SPTH or S2266. Both methods of inhibition rate constant measurements gave similar results. The ENZFITTER computer program (R. J. Leatherbarrow, Elsevier, Biosoft) was used for data analysis.

RESULTS

Thrombin inhibition by PCI was examined in the presence and absence of saturating levels of rabbit TM containing chondroitin sulfate and recombinant human TM fragment containing only the epidermal growth factor-like repeats 4–6 and lacking the chondroitin sulfate by a discontinuous assay method as shown in Fig. 1. The rate of inactivation of the bound thrombin was increased dramatically by both forms of TM. The acceleration of inhibition by rabbit TM was approximately 2–3 times more effective than TM 4–6 (Fig. 1 and Table I). These results indicate that, unlike TM-dependent acceleration of the inhibition of thrombin with antithrombin (16, 18), the TM-dependent acceleration of thrombin inhibition by PCI involves primarily protein-protein interactions rather than glycosaminoglycan-protein interactions.

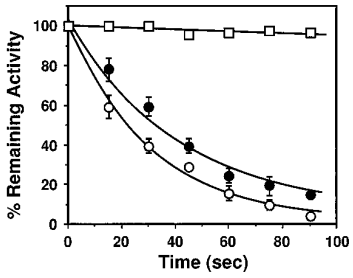

FIG. 1. **Time course of thrombin inhibition by PCI in the absence or presence of TM and TM 4–6.** Thrombin (0.5 nM) was incubated with PCI (10 nM) in the absence (□) or presence of 50 nM TM (○) or 100 nM TM 4–6 (●) in TBS buffer containing 2 mg/ml bovine serum albumin. At indicated time points, chromogenic substrate SPTH in TBS containing 1 mg/ml Polybrene was added to a final concentration of 0.2 mM, and, after the color development, the remaining amidolytic activity of uninhibited thrombin was determined as described under "Experimental Procedures." *Solid lines* were obtained by nonlinear regression analysis of data obtained from the average of three experiments using a first order rate equation.

In the presence of TM, the rate of inhibition was too fast to allow accurate assessment of the PCI concentration dependence of thrombin inhibition. As an alternative, the continuous assay method in the presence of SPTH as the competing chromogenic substrate was used to demonstrate the rate enhancement by TM and TM 4–6 with various concentrations of PCI. As shown in Fig. 2, in the presence of TM, inhibition was rapid, inhibitor concentration-dependent, and complete. Fig. 3 shows that the k' values are linear with PCI concentration. Note that with 100 nM PCI, virtually all thrombin activity was inhibited within 500 s. Similar results were obtained with TM 4–6 (data not shown). In contrast, in the absence of TM, the same concentration of PCI (100 nM) with SPTH as the competing chromogenic substrate failed to inhibit thrombin effectively (data not shown). In the absence of TM, S2266 was used as the competing chromogenic substrate to estimate the rate constants by the continuous assay method (Table I).

To allow comparisons of reaction rates, the k_2 values for thrombin inhibition by PCI in the absence and presence of TM or TM 4–6 were determined. These values were determined by both the discontinuous and continuous assay methods from inhibition progress curves in the presence of competing substrate as described under "Experimental Procedures." For the discontinuous assay, 17 independent inhibition reactions were performed in the presence of rabbit TM and 12 reactions in the presence of TM 4–6. The k_2 values determined by the discontinuous assays are given in the top lines of each section of Table I, and the values in the presence of competing substrate are given in the middle line of each section. In most cases, the values obtained by these two assay methods agreed within a factor of 2. Therefore, to simplify presentation of the influence of TM on the inhibition rate, the k_2 values were averaged, and the average value was used to calculate the fold enhancement by TM. The average value is given in the third line of each section of Table I.

Comparison of the rate constants in Table I reveals that TM 4–6 accelerates thrombin inhibition by PCI approximately 59-fold. Rabbit TM containing the chondroitin sulfate is only 2–3 times more effective. Taken together, these results indicate

TABLE I
The second order association rate constants of PCI and PCI F353P mutant inhibition of thrombin

	PCI	PCI F353P
	$M^{-1}\ s^{-1}$	$M^{-1}\ s^{-1}$
Thrombin	$2.0 \pm 0.3 \times 10^{4a}$	$9.6 \pm 1.7 \times 10^{5}$
	$1.4 \pm 0.1 \times 10^{4b}$	3.0×10^{5c}
Average	1.7×10^{4d}	6.3×10^{5}
Thrombin-TM	$2.7 \pm 0.8 \times 10^{6}$	$1.3 \pm 0.8 \times 10^{7}$
	$2.0 \pm 0.8 \times 10^{6}$	$6.9 \pm 1.5 \times 10^{6}$
Average	2.4×10^{6}	1.0×10^{7}
Thrombin-TM 4–6	$1.2 \pm 0.3 \times 10^{6}$	$7.1 \pm 2.4 \times 10^{6}$
	$8.1 \pm 0.8 \times 10^{5}$	4.5×10^{6c}
Average	1.0×10^{6}	5.8×10^{6}

[a] The values in the top lines are the average of more than 10 independent measurements with ±S.D. determined by the discontinuous assay method.
[b] The values in the middle lines are the average of 2–6 independent measurements with ± S.D. determined by the continuous assay by analysis of the progress curves in the presence of competing chromogenic substrate as described under "Experimental Procedures."
[c] Single measurements.
[d] The average k_2 values from two methods of measurements used in comparisons are shown in the third line.

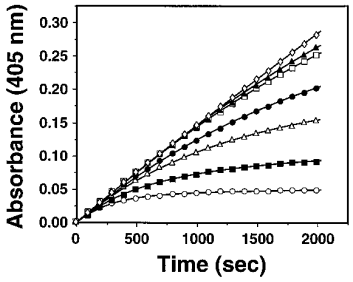

FIG. 2. **Typical progress curves for inhibition of thrombin by PCI in the presence of TM.** Thrombin (0.5 nM) in complex with TM (50 nM) was added to reactions containing 0.2 mM SPTH and varying concentrations of PCI in TBS buffer containing 2 mg/ml bovine serum albumin. The concentrations of PCI in reactions were: 0 (◇), 3.1 nM ( ), 6.3 nM (□), 12.5 nM (●), 25 nM ( ), 50 nM (■), and 100 nM (○). The pseudo-first order association rate constant (k') for inhibition was determined by fitting the data to Equations 2 and 3 (only every 100-s values are plotted).

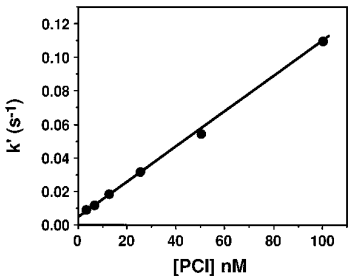

FIG. 3. **Linear dependence of k' values *versus* the concentration of PCI.** The pseudo-first order association rate constants of Fig. 2 are plotted *versus* the concentration of PCI. The slope of the straight line represents the second order association rate constant of inhibition.

that the protein-protein interactions are the most important contribution to the acceleration of thrombin inhibition by PCI.

The potential mechanisms by which the protein-protein interactions between TM and thrombin might augment PCI inhibition were examined by analyzing thrombin inhibition by a PCI mutant in the presence and absence of TM 4–6. The sequence of PCI from the P3 to the P3′ residues in the reactive center is Thr-Phe-Arg-Ser-Ala-Arg. Previous kinetic studies have illustrated that Phe in the P2 position fits poorly into the S2 specificity pocket of thrombin (26, 27) in contrast to Pro which is ideally suited to fit into this pocket (28). Mutation of Phe^{353} (the P2 residue) to Pro resulted in a PCI mutant that inhibited thrombin ≈37-fold better ($k_2 = 6.3 \times 10^5\ M^{-1}\ s^{-1}$) than wild type PCI. TM 4–6 enhanced inhibition by this mutant only 9-fold ($k_2 = 5.8 \times 10^6\ M^{-1}\ s^{-1}$) *versus* the 59-fold enhancement for wild type PCI suggesting that interaction of TM 4–6 with thrombin allowed thrombin to accommodate the larger and more hydrophobic Phe residue.

DISCUSSION

The observation that PCI reacts rapidly with thrombin in complex with TM suggests that physiologically PCI functions primarily to augment coagulation reactions and does so by inhibiting both protein C activation and APC itself. The rate of thrombin-TM complex inhibition by PCI is considerably more rapid than the rate of inhibition of free thrombin by antithrombin even allowing for the higher concentrations of antithrombin in the circulation (88 nM *versus* 2.3 μM). At room temperature, the calculated time to inhibit 50% of the thrombin bound to TM with plasma levels of PCI would be 3–4 s and with antithrombin would be 30 s. On thrombin-TM complexes that contain chondroitin sulfate, antithrombin may contribute more significantly to inhibition (calculated half-life, based on literature values, would be 2–6 s) (16, 17). In cell culture and probably *in vivo*, the addition of chondroitin sulfate to human thrombomodulin appears to be incomplete (29). With chondroitin-free TM, PCI is likely to be a major inhibitor of the complex. In addition to cell-associated chondroitin-free forms of TM, soluble TM generated by elastase proteolysis lacks the chondroitin sulfate, and soluble forms of TM are found at moderately high levels in patients with vascular diseases or inflammatory conditions (30). PCI may play a major role in inhibiting thrombin bound to these different forms of TM.

PCI is synthesized in the liver, testis, prostate, and kidney (31, 32). It is of interest that TM has been observed on several cell types not in contact with blood (33, 34). Given the tissue distribution of PCI, it is possible that PCI plays a role in inhibition of thrombin-TM complexes in the extravascular space. Examination of alternative sites of synthesis of this inhibitor may provide additional insights into the physiological function of the inhibitor.

At a biochemical level, these studies draw clear distinctions between the requirements for inhibition of thrombin bound to TM by the two serpins, antithrombin and PCI. With PCI, occupancy of anion-binding exosite 1 by TM 4–6 is sufficient for acceleration of inhibition by PCI with the chondroitin contributing only a 2–3-fold additional acceleration. In contrast, TM 4–6 or full-length TM devoid of the chondroitin sulfate fail to accelerate thrombin inhibition by antithrombin (16, 18). This suggests that TM-dependent acceleration of thrombin inhibition by PCI is largely dependent on conformational changes in thrombin resulting from the protein-protein interactions between thrombin and TM.

The mechanisms involved in allowing PCI to react rapidly with the thrombin-TM complex are likely to be due to the conformational changes that occur during complex formation in the extended binding pocket of thrombin. Comparison of TM 4–6 acceleration of thrombin inhibition by the wild type PCI (Phe at the P2 position) with the mutant PCI with Pro at the P2 position revealed that the TM 4–6 acceleration fell from ≈59-fold to ≈9-fold. We interpret these data to suggest that TM 4–6 allows thrombin to accept bulkier and/or more hydrophobic residues at the P2 position. Based on modeling and kinetic data, Phe would not fit well into the S2 pocket of thrombin in the absence of TM (26, 27). TM could allow thrombin to interact with Phe more favorably if, for instance, TM elicited conformational changes in the large insertion loop (the 60 loop) that forms the upper portion of the S2 specificity pocket (28). Pre-

vious studies examining inhibition of thrombin by bovine pancreatic trypsin inhibitor have suggested that this loop can exist in multiple conformations (35).

The observation that protein-protein interactions between thrombin and TM accelerate reaction with PCI represents another example of significant enhancement of thrombin reactivity with naturally occurring protein substrates and inhibitors. The other examples are protein C activation and single-chain urokinase-type plasminogen activator inactivation (36, 37). The exact mechanism of single-chain urokinase-type plasminogen activator inactivation has not been studied in depth, but protein C activation, like the inhibition of thrombin by PCI, appears to involve a conformational change in the active center of thrombin that overcomes interactions with residues that do not interact well within the extended binding pocket of thrombin in the absence of TM (38). The data presented here in combination with previous studies suggest that the S3, S2, and S3′ pockets of thrombin are all altered by interaction with TM. The observation that reactivity of PCI with thrombin is selectively altered by interaction with TM opens new approaches for investigating the molecular mechanisms by which TM switches the specificity of thrombin from a clot-promoting to a clot-inhibiting enzyme.

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