Thrombomodulin Increases the Rate of Thrombin Inhibition by BPTI†

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ABSTRACT: Thrombin undergoes allosteric modulation by thrombomodulin (TM) that results in a shift in macromolecular specificity, blocking fibrinogen clotting while enhancing protein C activation. The TM enhancement of protein C activation involves both an 8-fold decrease in K_m and a 200-fold increase in k_{cat}. Although TM-mediated conformational changes in thrombin have been detected by many techniques, the nature of these changes remains obscure. Access to the active center of thrombin is relatively restricted due to the presence of a large insertion loop at residue 60 (chymotrypsin numbering) that has been implicated in modeling studies as being responsible for poor inhibition by BPTI. Thrombin and the E192Q mutant, which binds BPTI much more tightly than thrombin, are both inhibited very slowly by BPTI. TM increases the rate of thrombin or thrombin E192Q inhibition by BPTI ∼10-fold. When analyzed as slow tight binding inhibition, the TM effect on thrombin E192Q inhibition by BPTI is primarily on the first, reversible step in the reaction. Structural studies of the thrombin E192Q−BPTI complex have previously shown that the 60 loop lies over the BPTI, a position which requires 8 Å movement at the apex of the 60 loop, and that BPTI is found in the same canonical orientation as in the trypsin complex. It follows that TM enhancement of the initial interaction of thrombin results in a conformation that favors interactions with BPTI, probably involving motion of the 60 loop.

The presence of two large insertion loops in thrombin relative to trypsin distinguishes thrombin from most other serine proteases (Stubbs & Bode, 1993). One of these insertion loops, the 60 loop, dramatically restricts access of macromolecules to the active site of thrombin. One of the best examples of steric restrictions relates to the inability of Kunitz inhibitors such as bovine pancreatic trypsin inhibitor (BPTI)[1] to inhibit thrombin. Modeling studies predict that there would be several collisions between BPTI and thrombin, particularly Tyr35 of BPTI with Trp60D in thrombin. Trp60D is largely responsible for restricting the access of macromolecules into the active site pocket (Bode et al., 1992a,b). The observation that the location of the 60 loop is similar in all published crystal structures suggested that this loop was rather inflexible. Consistent with the molecular modeling, deletion of Pro60B, Pro60C, and Trp60D resulted in a thrombin mutant, thrombin des PPW, that is inhibited effectively by BPTI, but this mutant is a very poor enzyme toward any natural substrate and has impaired activity toward chromogenic substrates (Le Bonniec et al., 1993).

The possibility that the 60 loop is more mobile than previously hypothesized was suggested by the observation that isosteric substitution of Glu192 in thrombin with Gln, thrombin E192Q, results in a mutant which binds BPTI tightly (Guinto et al., 1994; Rezaie & Esmon, 1996). For BPTI to interact with thrombin E192Q in a canonical fashion, the loop would have to move, and hence significant flexibility in the loop was proposed (Guinto et al., 1994). Less likely alternatives were that inhibition might involve some major alteration in BPTI conformation or the mechanism of BPTI−thrombin inhibition. Structural analysis of the BPTI−thrombin E192Q complex demonstrated that the conformation of BPTI with thrombin E192Q is virtually identical to that observed with trypsin and that Trp60D at the apex of the loop undergoes an 8 Å displacement in forming the BPTI thrombin−E192Q complex (van de Locht et al., 1997). The 60 loop, and Trp60D in particular, makes many contacts with BPTI probably contributing to the stability of the final complex.

Thrombin is subject to regulation by a variety of ligands. Of these, perhaps the most dramatic differences exist between free thrombin and the thrombin−thrombomodulin (TM) complex. TM binding to thrombin blocks fibrinogen clotting and platelet activation while promoting protein C activation and inhibition by antithrombin and the protein C inhibitor (Esmon, 1993; Rezaie et al., 1995). Enhancement of antithrombin inhibition is dependent on the presence of a chondroitin sulfate moiety on TM. This moiety is not essential for protein C activation or enhanced inhibition by

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[1] Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; thrombin E192Q, a thrombin mutant in which Glu192 [in the chymotrypsin numbering system of Bode et al. (1989)] is substituted with Gln; TM, thrombomodulin; PEG, polyethylene glycol; SPTH, Spectrozyme TH.

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protein C inhibitor, but does alter thrombin conformation (Ye et al., 1993), changes several kinetic parameters of protein C activation (Liu et al., 1994), and further enhances thrombin inhibition by the protein C inhibitor (Rezaie et al., 1995). Available structural and functional data indicate that the EGF-like repeats 5−6 of TM bind to anion binding exosite 1 of thrombin (Ye et al., 1992) and that the fourth repeat exits thrombin in the general direction of the 60 loop (Mathews et al., 1994b). Although TM EGF 5−6 bind to thrombin with equal affinity to TM 4−6, TM 4−6 is required for optimal protein C activation. TM decreases the K_m 8-fold and increases k_{cat} 200-fold (Galvin et al., 1987; Esmon et al., 1983). Attempts to mimic the TM effect on K_m by loop deletion have resulted in the formation of thrombin derivatives with low proteolytic activity (Le Bonniec et al., 1992, 1993). Kinetic studies have indicated that Trp60D, the Trp residue largely responsible for steric hindrance of the active site with BPTI, plays an inhibitory role in the initial docking of antithrombin to thrombin but is involved in stabilizing the tight complex (Rezaie, 1996). Given the prominent roles of the 60 loop in regulating substrate/inhibitor access to the active center of thrombin, we felt that modulation of the conformation of this loop might play a major role in the mechanisms by which TM augments protein C activation. Based on the concept that BPTI inhibition of thrombin is slow and weak due to steric restriction of the active site to BPTI, we reasoned that measurements of the rate of BPTI inhibition of thrombin might provide a monitor of motion in the 60 loop facilitated by TM interaction.

In this study, we demonstrate that TM enhances the rate of thrombin or thrombin E192Q inhibition by BPTI. With thrombin E192Q, this enhancement results from increased affinity of the initial complex rather than accelerating formation of the tight complex. Since the structural analysis revealed that the 60 loop must be distorted for complex formation to occur, the observation that TM enhances the rate of complex formation with BPTI is consistent with the concept that TM increases access to the active center of thrombin, probably by moving the 60 loop toward the conformation observed in the BPTI−thrombin E192Q complex.

MATERIALS AND METHODS

Expression, purification, and active site titration of thrombin E192Q and plasma-derived wild-type human thrombin were previously described (Guinto et al., 1994). Soluble TM fragments containing or lacking the chondroitin sulfate were prepared as described (Liu et al., 1994). Spectrozyme TH (SPTH) and hirudin synthetic C-terminal dodecapeptide (*N*-acetyl-Hir-53′-64′-OH with sulfato-Tyr63′, product 5343) were purchased from American Diagnostica, Greenwich, CT; S2266 was purchased from Kabi Pharmacia/Chromogenix, Franklin, OH; and BPTI was purchased from Sigma.

Kinetic Analysis. The inhibition constant (K_i) for BPTI inhibition of wild-type thrombin was determined by both continuous and discontinuous assay methods in the absence and presence of TM containing chondroitin sulfate. In the continuous assay, 0.5−1 nM thrombin alone (final concentration) was added to wells of a 96-well polystyrene plate containing various concentrations of BPTI (0−125 μM) and 100 μM S2266 as the competing chromogenic substrate, and the residual enzyme activity was measured for 1 h at room temperature at 405 nm with a Vmax Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA). The buffer consisted of 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.5, containing 1 mg/mL BSA and 0.1% polyethylene glycol 8000 (PEG 8000) to minimize thrombin adsorption. In the discontinuous assay, 7.5 nM thrombin in the absence or presence of 100 nM TM was preequilibrated with various concentrations of BPTI (0−125 μM) in the same buffer for 1 h at room temperature, and then chromogenic substrate S2266 (200 μM) was added and the residual enzyme activity was measured as described above. The K_i values were estimated by nonlinear regression analysis using eq 1 as described (Rezaie & Esmon, 1993, 1996):

$$v_s = v_o/\{[I]/K_i(1 + [S]/K_m) + 1\} \quad (1)$$

where v_s and v_o are the steady-state velocities of chromogenic substrate hydrolysis in the presence and absence of inhibitors, respectively, [S] is the chromogenic substrate concentration, K_m is the Michaelis−Menten constant, and [I] is the total inhibitor concentration. The K_m values for S2266 hydrolysis by thrombin were 217 and 156 μM in the absence and presence of TM, respectively. Time course analysis for a duration of 5 h indicated that equilibrium is established after 30 min and that the determined K_i values remain essentially identical whether thrombin and BPTI are incubated for 30 min or 5 h, prior to addition of chromogenic substrate. In both assays, following the addition of S2266, it was ensured that less than 10% chromogenic substrate was hydrolyzed, and only data collected after attainment of the new equilibrium were used to calculate the inhibition constants.

Slow Binding Kinetic Analysis. The slow binding kinetic approach was employed to determine the K_i values for thrombin E192Q inhibition (Morrison & Walsh, 1988). In this case, a series of inhibition progress curves were generated by adding 0.5−1 nM enzyme alone (final concentration) or in complex with 100 nM TM containing chondroitin sulfate to wells of a 96-well polystyrene plate containing various concentrations of BPTI (0−10 μM in the absence of TM, and 0−800 nM in the presence of TM) and 250 μM competing chromogenic substrate S2266. Data from each curve at different inhibitor concentrations were fit by nonlinear regression analysis using the Enzfitter computer program (R. J. Leatherbarrow, Elsevier, Biosoft) to the integrated rate equation for slow binding inhibition (Morrison & Walsh, 1988):

$$A = v_s t + (v_o - v_s)(1 - e^{k_{obs}t})/k_{obs} + A_o \quad (2)$$

where A is the absorbance at 405 nm at time t, v_o and v_s are the initial and final steady-state velocity, respectively, k_{obs} is the apparent first-order rate constant, and A_o is the initial absorbance at 405 nm. Fitting gave values for v_o, v_s, k_{obs}, and A_o for each progress curve. These values were analyzed by different methods to obtain inhibition and reaction rate constants (see below). The K_m values for S2266 hydrolysis by thrombin E192Q at room temperature in TBS containing 1 mg/mL BSA, 0.1% PEG 8000 were 298 and 223 μM in the absence and presence of TM, respectively.

Dissociation of Preformed Complexes. For direct estimation of the rate constant for the dissociation of thrombin

E192Q−BPTI complex in the absence or presence of TM, enzyme−inhibitor complexes were preformed by incubating thrombin E192Q with BPTI at a final concentrations of 1 μM enzyme and 5 μM inhibitor in the absence and presence of 2 μM TM containing chondroitin sulfate in 20 μL reactions for 30 min at room temperature. The complex was then diluted 1.6×10^4-fold into the TBS buffer described above, containing 200 μM SPTH. The regeneration of amidolytic activity was monitored at 2-min intervals for 4 h. Data were fitted to eq 2 to obtain the estimate for the k_{obs} of the reactions. Less than 10% chromogenic substrate was hydrolyzed in the reactions. The first-order rate constant, k_4, for dissociation of enzyme was determined from eq 3 as described by Morrison and Walsh (1988):

$$k_{obs} = k_4\{(1 + [I])/(K_i^*(1 + [S]/K_m))/ (1 + [I])/(K_i(1 + [S]/K_m))\} \quad (3)$$

K_i* and K_i represent the final and initial inhibition constants, respectively (see Results). The K_m for SPTH hydrolysis by thrombin E192Q was 10 μM both in the presence and in the absence of TM.

Inhibition Time Course. The time course of inhibition of thrombin E192Q with BPTI was measured as follows. Thrombin E192Q (5 nM) was incubated with BPTI (500 nM) at room temperature (21−25 °C), in the buffer system described above, in the presence or absence of TM (200 nM) that did or did not contain chondroitin sulfate. Samples were removed at the times indicated, and residual thrombin activity was monitored by addition of SPTH to a final concentration of 200 μM by adding 20 μL of a 2 mM stock solution to 100 μL reactions. Addition of SPTH did not cause rapid dissociation of the enzyme−inhibitor complex since the rates were linear for the 10 min of the assay. Furthermore, increasing the concentration of SPTH (25−400 μM) did not influence the residual activity of thrombin, suggesting that the enzyme−inhibitor complex dissociates slowly.

RESULTS

Thrombin is well-known to be resistant to inhibition by BPTI, in part because of the surface loops that restrict access to the catalytic center. Several loops on the surface of thrombin undergo structural rearrangement following complex formation with BPTI. The magnitude and nature of these changes, which are important for understanding the experiments that follow, are illustrated in Figure 1. In addition to major changes in the location of the 60 loop, the 99, 148, and 39 loops also undergo rearrangements, suggesting that these loops may be allosterically linked.

Previous spectroscopic and mutagenesis studies indicated that TM binding to thrombin induces a conformational change in the active site pocket that changes the macromolecular substrate and inhibitor specificity of thrombin (Ye et al., 1992; Le Bonniec & Esmon, 1991; Rezaie et al., 1995; Rezaie, 1997). To determine whether complex formation with TM influences the affinity of thrombin for BPTI, the K_i values were determined in the presence or absence of TM by two assay methods described under Materials and Methods. The results of both assays were similar, and they indicated that BPTI inhibits the thrombin−TM complex better than thrombin alone (Figure 2) with the K_i values of

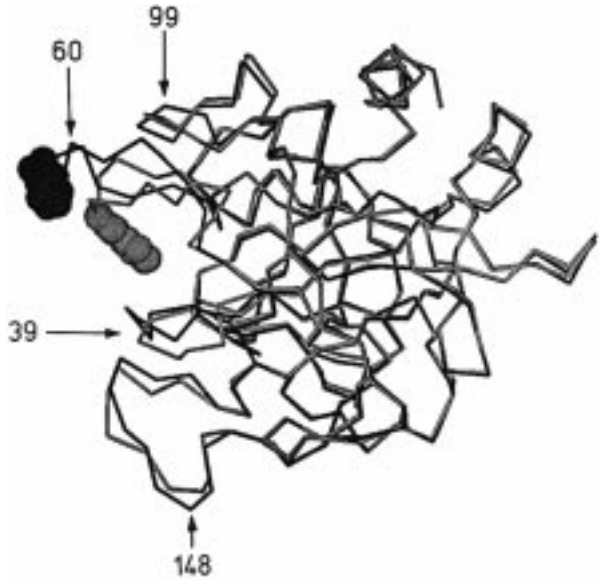

FIGURE 1: α-Carbon structure of thrombin inhibited with D-Phe-Pro-Arg chloromethyl ketone (gray) superimposed with thrombin E192Q inhibited by BPTI (black). Trp60D is shown by space-filling. The structures are rotated 90° to the left from the standard orientation. The numbered arrows refer to the insertion loops discussed in the text that undergo conformational rearrangements upon interaction with BPTI.

55.9 ± 0.9 and 11.9 ± 0.3 μM in the absence and presence of TM, respectively.

We previously demonstrated that isosteric substitution of Glu192 with Gln in thrombin results in mutant (E192Q) which binds BPTI with a K_i value that is at least 1000-fold lower than that of wild-type thrombin (Guinto et al., 1994; Rezaie & Esmon, 1996). Since thrombin E192Q binds TM normally (Le Bonniec & Esmon, 1991) and one possible mechanism by which TM might modulate thrombin function is to increase access to the catalytic center of thrombin by moving these loops, we decided to examine the influence of TM on thrombin E192Q inhibition by BPTI by the slow binding kinetic approach. BPTI inhibition of E192Q thrombin was studied in a continuous assay in the presence of S2266 as the competing chromogenic substrate. A series of progress curves were generated with different concentrations of inhibitor. As shown in Figure 3A, BPTI inhibition of thrombin E192Q was of the slow, tight binding type inhibition mechanism as described by Morrison and Walsh (1988). The k_{obs} values were determined by fitting data to the slow binding eq 2. The plot of k_{obs} values as a function of BPTI concentration for thrombin E192Q in the absence and presence of TM was determined and was hyperbolic in both cases. The curve in the presence of TM is shown in Figure 3B. The hyperbolic shape of the curve is compatible with the inhibition mechanism shown in Scheme 1 in which inhibitor (I) and enzyme (E) form an initial reversible complex with a $K_i = k_2/k_1$, followed by a slow transition to a tight complex, EI*.

Scheme 1

$$E \underset{k_2}{\overset{k_1}{\leftrightarrow}} EI \underset{k_4}{\overset{k_3}{\leftrightarrow}} EI^*$$

In this case, the initial K_i (k_2/k_1), k_3, k_4, and the final K_i* are estimated from the following equations (Huang et al.,

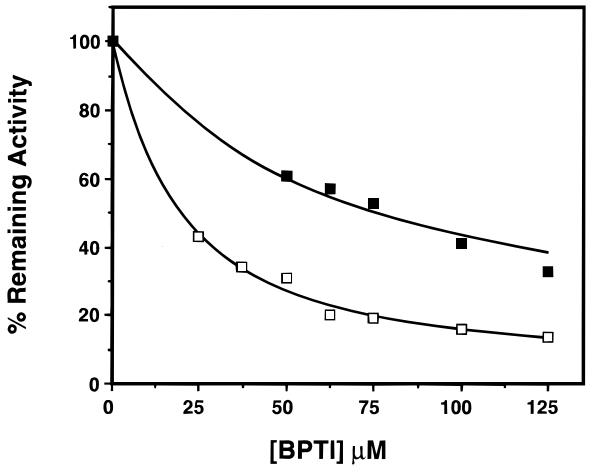

FIGURE 2: Influence of thrombomodulin on BPTI inhibition of wild-type thrombin. Thrombin (1 nM) alone (■), or in complex with 100 nM TM containing chondroitin sulfate moiety (□), was incubated with 100 μM S2266 and the indicated concentrations of BPTI in TBS buffer containing 1 mg/mL BSA and 0.1% PEG 8000 at room temperature as described under Materials and Methods. The solid lines are nonlinear regression fits of the residual thrombin activities to eq 1.

1993; Morrison & Walsh, 1988):

$$k_{obs} = k_4 + k_3[I]/([I] + K_i(1 + [S]/K_m)) \quad (4)$$

$$k_4 = k_{obs}v_s/v_o \quad (5)$$

$$K_i^* = K_i k_4/(k_3 + k_4) \quad (6)$$

The K_i, k_3, k_4, and K_i^* values for BPTI inhibition of thrombin E192Q in the absence and presence of TM are listed in Table 1. TM lowered the K_i^* value ~10-fold for thrombin E192Q inhibition by BPTI primarily by influencing the initial, reversible step of the reaction (Table 1).

The value of k_4 was also determined directly from the rate of dissociation of preformed thrombin E192Q−BPTI complex by excessively diluting the inhibitory complex into a 200 μM solution of the competing chromogenic substrate, SPTH, which has a low K_m for thrombin E192Q (10 μM). As shown in Figure 4, the amidolytic activity of thrombin E192Q was recovered as it slowly dissociated from the inhibitory complex. Under the dilution conditions, no reassociation of the inhibitor with the enzyme would occur since the inhibitor concentration is ~4704-fold less than $K_{i(app)}^*$ in the absence of TM and ~490-fold less than $K_{i(app)}^*$ in the presence of TM [where $K_{i(app)}^* = K_i^*(1 + [S]/K_m)$]. Under these conditions, the value of the term $\{(1 + [I])/(K_i^*(1 + [S]/K_m))/(1 + [I])/(K_i(1 + [S]/K_m))\}$ in eq 3 reaches unity, and therefore, $k_{obs} \approx k_4$. Using this approach, the k_4 values were determined to be $(1.9 \pm 0.6) \times 10^{-4}$ s^{-1} in the absence of TM, and $(1.8 \pm 0.6) \times 10^{-4}$ s^{-1} in the presence of TM ($n = 4$, ±SD), which are in close agreement with the values listed in Table 1 supporting the validity of the measurements. Increasing either the inhibitor or the chromogenic substrate concentration 2-fold did not change the k_4 values further, suggesting that the reassociation of enzyme and inhibitor is not a factor in these measurements.

Inhibition Time Course Analysis. With BPTI inhibition of thrombin E192Q, the apparent extent of inhibition was not altered by altering SPTH concentration from 25 to 400 μM during analysis of the residual enzyme activity (10 min), suggesting that the inhibition is not readily reversible under the experimental conditions. Based on this observation, we felt that if the inhibition is slow enough, a simple time course analysis is valid to monitor the effect of TM on BPTI inhibition of thrombin E192Q. Consistent with this, as shown in Figure 5, TM also enhanced the rate of inactivation of thrombin E192Q by BPTI about 10-fold by this method of analysis. The rate of inhibition by BPTI was enhanced by the presence of the chondroitin sulfate moiety on TM, although both forms increased the rate of inhibition (compare the top and middle lines in Figure 5). With wild-type thrombin, addition of TM to thrombin also increased the rate of BPTI inhibition ~10-fold (the half-maximal inhibition of thrombin in the absence and presence of TM occurred after ~16 min and 1.5 min, respectively); however, the presence of chondroitin sulfate on TM did not influence the rate of wild-type thrombin inhibition (data not shown).

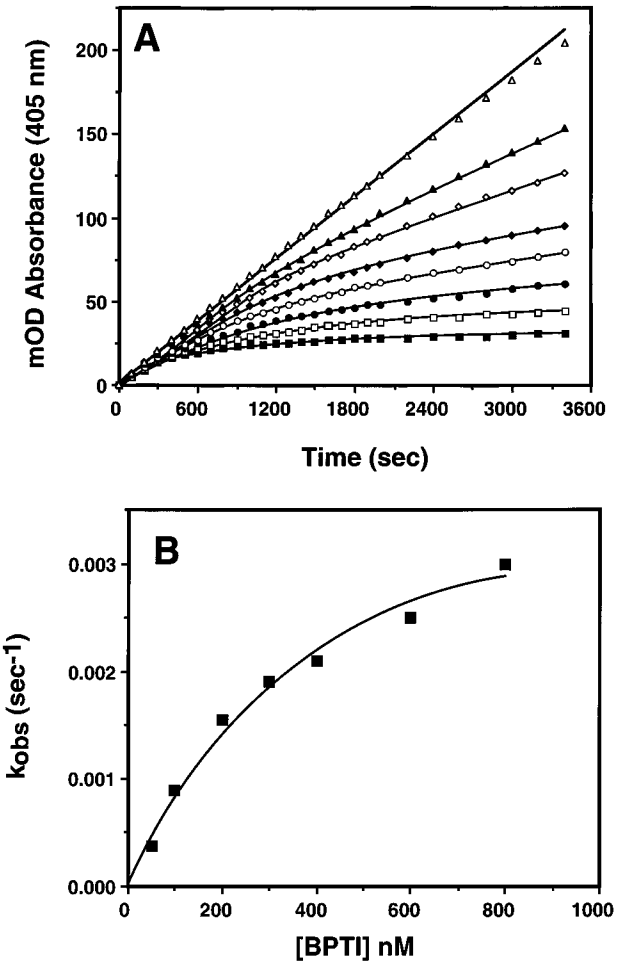

FIGURE 3: Progress curves for BPTI inhibition of thrombin E192Q. (A) Thrombin E192Q (0.5 nM) in complex with 100 nM TM containing chondroitin sulfate was added to reactions containing 250 μM S2266 and varying concentrations of BPTI in TBS buffer containing 1 mg/mL BSA and 0.1% PEG 8000. The concentrations of BPTI in reactions were 0 (△), 50 nM (▲), 100 nM (◇), 200 nM (◆), 300 nM (○), 400 nM (●), 600 nM (□), and 800 nM (■). The pseudo-first-order association rate constant (k_{obs}) for inhibition was determined by fitting the data to eq 2. (B) Dependence of k_{obs} on BPTI concentration with thrombin E192Q in the presence of TM. The k_{obs} values in the presence of TM were fitted to eq 4, a hyperbolic relationship that describes the dependence of k_{obs} as a function of inhibitor concentration for a two-step reaction mechanism described by Scheme 1 under Results.

Table 1: Kinetic Parameters for BPTI Inhibition of Thrombin E192Q in the Absence and Presence of TM[a]

	K_i (M)	k_3 (s^{-1})	k_4 (s^{-1})	K_i*
E192Q (−TM)	$(1.1 \pm 0.5) \times 10^{-6}$	$(3.1 \pm 0.1) \times 10^{-3}$	$(2.1 \pm 0.4) \times 10^{-4}$	$(7.0 \pm 3.2) \times 10^{-8}$
E192Q (+TM)	$(1.4 \pm 0.2) \times 10^{-7}$	$(3.1 \pm 0.6) \times 10^{-3}$	$(1.7 \pm 0.2) \times 10^{-4}$	$(7.3 \pm 0.7) \times 10^{-9}$

[a] Thrombin E192Q (0.5 nM) inhibition by BPTI (0−10 μM in the absence of TM, and 0−800 nM in the presence of 100 nM TM) was continuously monitored in the presence 250 μM S2266 in TBS buffer containing 1 mg/mL BSA and 0.1% PEG 8000 at room temperature as described under Materials and Methods. The inhibition constants were determined from eqs 2−6 as described in the text.

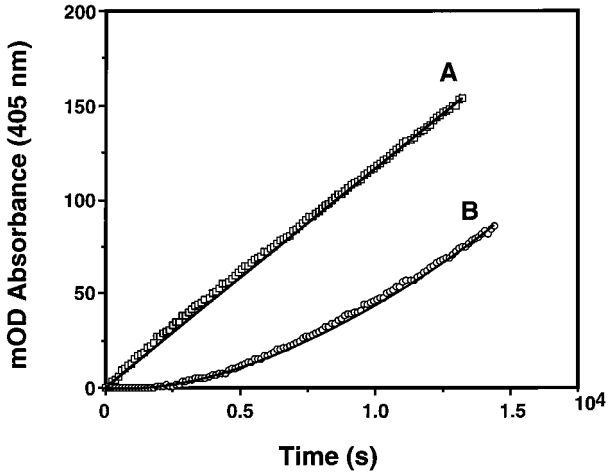

FIGURE 4: Dissociation of thrombin E192Q−BPTI preformed complex. An aliquot of preformed thrombin E192Q (1 μM) and BPTI (5 μM) complex was diluted 1.6×10^4-fold into 200 μM SPTH in TBS buffer containing 1 mg/mL BSA and 0.1% PEG 8000 as described under Materials and Methods. Dissociation of enzyme from the inhibitory complex was monitored by the regeneration of the amidolytic activity as measured at 405 nm. Curve A is the activity of enzyme in the absence of BPTI, and curve B is the regeneration of enzyme activity from the enzyme−inhibitor complex. The value of k_4 was determined directly from fitting the data of curve B to eq 2 (solid line) as described under Results.

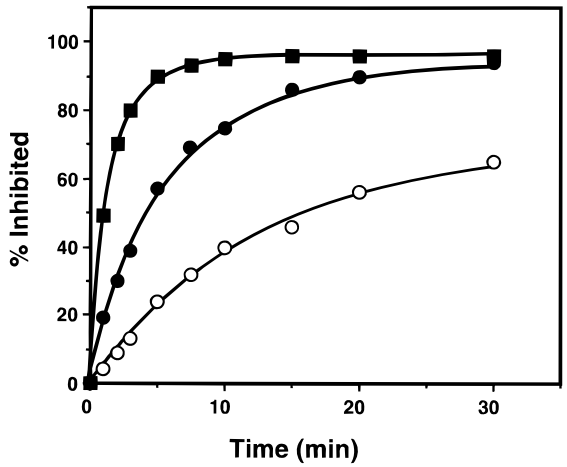

FIGURE 5: The influence of thrombomodulin on the time course of thrombin E192Q inhibition by BPTI. Thrombin E192Q (5 nM) alone (○) or in complex with 200 nM TM lacking (●) or containing chondroitin sulfate moiety (■) was incubated with BPTI (500 nM) in TBS buffer containing 1 mg/mL BSA and 0.1% PEG 8000 at room temperature. At the indicated time points, SPTH was added to a final concentration of 200 μM, and the remaining uninhibited thrombin activity was measured as described under Materials and Methods.

To ensure thrombin E192Q is saturated with TM under our experimental conditions, BPTI inhibition of thrombin E192Q in the presence of TM was monitored at four concentrations of 25, 50, 100, and 200 nM TM. The cofactor effect of TM was the same in all TM concentrations examined. Furthermore, to determine whether the exosite 1 occupancy of thrombin by TM accounts for the acceleration of inhibition, BPTI inhibition of thrombin E192Q was monitored in the presence of 25 nM TM and increasing concentrations (25−1600 nM) of hirudin C-terminal dodecapeptide which is known to bind the exosite 1 of thrombin (Rydel et al., 1990). The hirudin peptide inhibited the cofactor function of TM in a dose-dependent fashion. With 1600 nM hirudin C-terminal peptide, the cofactor function of TM in acceleration of thrombin inhibition by BPTI was completely abolished. Control experiments in the presence of 1600 nM hirudin C-terminal peptide alone suggested that the peptide by itself does not influence the K_i of thrombin E192Q inhibition by BPTI. These results suggest that TM occupancy of exosite 1 of thrombin is primarily responsible for the acceleration of thrombin inhibition by BPTI.

DISCUSSION

The insertion loops in thrombin are strategically located to restrict access of certain substrates to the active site of thrombin. The loops, however, also serve as active participants in the binding of other substrates and inhibitors (Bode et al., 1992a,b; Le Bonniec et al., 1993; Stubbs & Bode, 1993). In particular, the 60 loop forms the top of the S2 pocket accounting at least in part for the preference of thrombin for small hydrophobic residues at the P2 position (Le Bonniec et al., 1993).

The observation that TM increases the rate of BPTI interaction with thrombin is interpreted most easily in terms of minimizing the steric hindrance to the active site. This probably involves movement of the 60 loop which modeling studies have implicated as the major steric hindrance to BPTI access to the active center of thrombin. Support for this concept comes from the observation that partial deletion of the 60 loop enhances the on rate of BPTI (Le Bonniec et al., 1993). Furthermore, the Trp60D in the loop is distorted from its normal position by 8 Å in the BPTI−thrombin E192Q complex (van de Locht et al., 1997). Interestingly, the 60 loop forms a lid over the BPTI once the complex forms. Therefore, the loop retards the formation of the initial complex but stabilizes the complex once the initial contact is formed. Nearly 1200 Å of accessible surface is covered in the thrombin−BPTI complex compared to 800 Å in the trypsin−BPTI complex, illustrating the large number of additional interactions that are involved in the thrombin complex (van de Locht et al., 1997). This stabilization of the 60 loop probably accounts for the present finding that even though thrombin is poorly inhibited by BPTI, the complex is not readily dissociated by addition of high concentrations of substrate.

In addition to TM-mediated conformational changes in the 60 loop inferred from this study, TM probably causes other conformational changes in thrombin including residue 192 (Le Bonniec & Esmon, 1991), residue 39 (Le Bonniec et al., 1991), residue 99 (Rezaie, 1997), and the sodium binding site in thrombin (Wells & Di Cera, 1992), any or all of which might further modulate interaction with BPTI.

Previous studies have shown that changing Glu192 to Gln resulted in a thrombin mutant that activated protein C in the absence of TM much more rapidly than thrombin, but that in the presence of TM, the differences between the two thrombins were largely eliminated (Le Bonniec & Esmon, 1991). Crystal structures have shown that the residue 192 side chain conformation is sensitive to occupancy of anion binding exosite 1 (Mathews et al., 1994a). In the crystal structure of thrombin E192Q with BPTI, the carboxamide nitrogen atom of Gln192 hydrogen bonds to the backbone carbonyls of Cys14 and Gly12 of BPTI. The environment in this cavity is very electronegative and hence would not favor Glu192 from wild-type thrombin (van de Locht et al., 1997). The observation that TM increases inhibition at equilibrium with thrombin E192Q to a greater extent than wild-type thrombin would be consistent with TM shifting the conformation of residue 192 toward that seen in the BPTI−thrombin E192Q crystal, i.e., favorable for the mutant and less favorable for thrombin.

Another residue implicated in TM function is Glu39. In the BPTI−thrombin E192Q crystal, Glu39 is in a salt bridge with Arg20 of BPTI. The 39 loop is also distorted in the crystal structure (van de Locht et al., 1997). It is therefore possible that the motion in residue 39 inferred by mutational analysis to participate in TM function may also contribute to stabilizing TM enhancement of the BPTI−thrombin E192Q complex.

From the crystal structure, it is apparent that, in addition to the 60 and 39 loops, the 148 and 99 loops are distorted. The 148 loop is found in many different conformations in the crystal structures of various thrombin complexes that have been solved to date (Tulinsky & Qiu, 1993; van de Locht et al., 1997). It is unlikely that alteration in the 148 loop enhances the rate of BPTI binding since it does not appear to block access of BPTI to the active site of thrombin. The motion of the 148 loop could help stabilize the complex, however, since Trp148 is hydrogen bonded to Val34 in BPTI. Since all of the loops are distorted from their positions in previous structures when thrombin E192Q complexes with BPTI, it is likely that they are linked. Allosteric linkage through the sodium site has been proposed previously, although the exact regions affected by this site are unclear. Deletion of residues 146−148 within the 148 loop results in changes in the S2 binding pocket, possibly reflecting conformational changes in the 60 loop that forms part of the S2 pocket (Le Bonniec et al., 1994). It is possible, therefore, that TM-mediated changes in the 60 loop conformation result from interactions of TM with any of the loops mentioned above and the allosteric linkage is responsible for opening access to the active center of thrombin.

TM can exist as a proteoglycan with a chondroitin sulfate moiety (Bourin & Lindahl, 1993). Previous studies have implicated anion binding exosite 1 as the site on thrombin that mediates protein−protein interaction with TM and the heparin binding site (anion binding exosite 2) on thrombin as the binding site for chondroitin sulfate on TM (Ye et al., 1994; Sheehan & Sadler, 1994). Occupancy of either site alters the conformation of thrombin as monitored by fluorescent probes (Ye et al., 1992, 1993). In the present study, we observed that the chondroitin sulfate further accelerates BPTI inhibition of thrombin E192Q but has little influence on inhibition of wild-type thrombin. Chondroitin sulfate might favor the allosteric switch in thrombin since a cluster of basic residues (93, 97, and 101) located near the 99 loop have been implicated in chondroitin sulfate interaction (Ye et al., 1994). As noted above, the 99 loop undergoes significant displacement in the BPTI−thrombin E192Q structure, and since these may be linked conformational changes, the chondroitin interaction with this site might simply facilitate the conformational changes.

Motion within the 60 loop, especially motion that tends to open the loop, would be very consistent with the previous observation that TM allowed thrombin to be inhibited rapidly by the protein C inhibitor (Rezaie et al., 1995). In this case, the Phe at the P2 position is too large to be easily accommodated by the standard thrombin conformation normally observed in the crystal structures. Mutation of the unfavored Phe to the favored Pro at the P2 position resulted in loss of most of the capacity of TM to accelerate the inactivation of thrombin by the protein C inhibitor. The same mutation made the inhibitor much more effective in inhibiting free thrombin (Rezaie et al., 1995).

With respect to protein C activation, one of the features of this process that is most characteristic and unusual is that Ca^{2+} inhibits protein C activation by free thrombin (Amphlett et al., 1981) but is essential to rapid activation by the thrombin−TM complex (Esmon et al., 1983). With free thrombin, Ca^{2+} inhibition of protein C activation is largely due to an increase in K_m whereas the acceleration by Ca^{2+} with the thrombin−TM complex is due to a decrease in K_m and an increase in k_{cat} (Esmon et al., 1983). Although speculative, these observations would be consistent with the hypothesis that the Ca^{2+}-stabilized conformer of protein C is sterically excluded from the catalytic center of thrombin by the 60 loop and that TM, by moving the 60 loop, allows increased access of the Ca^{2+}-stabilized protein C conformation to the active center of thrombin. Deletion mutation to test this possibility was not successful since the resultant enzyme had very little activity toward protein C, possibly because the 60 loop could not form the "lid" for the substrate. Future structural determinations of the thrombin−TM complex and of protein C in the presence and absence of Ca^{2+} will be necessary to test these proposals.

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