Influence of Arginines 93, 97, and 101 of Thrombin to Its Functional Specificity†

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ABSTRACT: Mutation of three Arg residues, 93, 97, and 101, to Ala in thrombin (thrombin R93,97,101A) has previously been shown to eliminate most heparin acceleration of thrombin inhibition by antithrombin and most of the ability of chondroitin sulfate (CS) on thrombomodulin (TM) to enhance affinity for TM and to eliminate the characteristic high-affinity interaction with protein C observed with TM lacking CS. In this study we examined the relative impact of mutation of these Arg residues alone and in combination on the above reactions and, in addition, on the ability of rabbit TM to accelerate thrombin inhibition by antithrombin. The order of importance for heparin acceleration of inhibition by antithrombin was Arg 101, 93, and 97. In contrast, Arg 97 was the major residue required for TM-dependent acceleration of reactivity with antithrombin and for CS-dependent enhancement of TM affinity. Arg 101 and 93 were critical for TM-dependent, high-affinity protein C interaction at low Ca^{2+} concentrations, while Arg 97, which was critical for the other TM-dependent effects, played no detectable role in this metal dependence. These results illustrate that these Arg residues in anion binding exosite 2 contribute very differently to the diverse reactions dependent on that domain in thrombin.

Thrombin is a trypsin-like serine protease that both promotes and inhibits blood coagulation. As a coagulant enzyme, thrombin clots fibrinogen and activates platelets and factors XIII, V, and VIII (Walz et al., 1986; Tulinsky & Qiu, 1993; Stubbs & Bode, 1993). Thrombin bound to thrombomodulin (TM)[1] functions as an anticoagulant by activating protein C, which then inactivates factors Va and VIIIa to inhibit further thrombin generation (Dittman & Majerus, 1990; Parkinson et al., 1992; Sadler et al., 1993; Esmon, 1995). The thrombin/TM complex has impaired ability to clot fibrinogen and activate platelets or factors V, VIII, and XIII. The rate of thrombin inhibition by antithrombin and the protein C inhibitor is enhanced by TM (Rezaie et al., 1995). With antithrombin, this acceleration is dependent on the presence of a chondroitin sulfate moiety that can be covalently attached to TM during biosynthesis (TM+CS) (Preissner et al., 1987). Chondroitin sulfate on TM also increases the affinity for thrombin 10-fold (Ye et al., 1994). Heparin is another sulfated glycosaminoglycan that is involved in thrombin regulation. Heparin increases the rate of thrombin inhibition by antithrombin approximately 1000-fold through a mechanism that involves simultaneous interaction of heparin with thrombin and antithrombin. Antithrombin is believed to be the major physiological inhibitor of thrombin.

Structure−function studies and the crystal structure of thrombin in complex with the thrombin receptor peptide (Mathews et al., 1994a), hirudin (Rydel et al., 1991), a thrombin binding peptide from TM (Mathews et al., 1994b), and prothrombin fragment 2 (Arni et al., 1993) have combined to define sites of interaction of thrombin with macromolecules. These studies indicate that the thrombin receptor, a region of fibrinogen on the P′ side of the cleavage sites, and TM all interact with a deep groove in thrombin bordered on the upper edge by basic residues and referred to as anion binding exosite 1. Cross-competition studies coupled with the crystal structures described above indicate that TM blocks fibrinogen clotting, platelet activation, and factor V activation, at least in part, by overlapping with a portion of their binding site on thrombin (Hofsteenge et al., 1986; Ye et al., 1992; Wu et al., 1992; Esmon & Lollar, 1996). A second basic surface on thrombin, referred to as anion binding exosite 2, is located on nearly the opposite side of thrombin from exosite 1. Prothrombin fragment 2 binds to this site (Arni et al., 1993). When fragment 2 is bound to thrombin, heparin binding is impaired, and heparin cannot accelerate inhibition by antithrombin (Schoen & Lindhout, 1987; Liu et al., 1994). Three residues in anion binding exosite 2 are clustered relatively close together and make salt links to prothrombin fragment 2 (Arni et al., 1993). The combined observations that these residues are important in prothrombin fragment 2 binding and that fragment 2 can block heparin interaction with thrombin prompted us to

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[1] Abbreviations: TM, thrombomodulin; TM+CS, thrombomodulin containing chondroitin sulfate; TM−CS, thrombomodulin lacking chondroitin sulfate; thrombin R93A, R97A, and R101A, thrombin in which the Arg residues 93, 97, and 101 [in the chymotrypsin numbering system of Bode et al. (1989)] are converted to Ala; PC-PT Gla, a chimeric protein C molecule in which the Gla domain and the hydrophobic stack region of protein C (exons 2 and 3) are replaced by the corresponding regions of prothrombin; AT, antithrombin; MES, 2-(*N*-morpholino)ethanesulfonic acid; BHK, baby hamster kidney; BSA, bovine serum albumin.

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mutate these residues to determine if they were critical to glycosaminoglycan interactions with thrombin. Mutation of these three Arg residues to Ala resulted in almost complete elimination of heparin affinity, the ability of heparin to accelerate antithrombin inhibition, and the ability of the chondroitin sulfate moiety to enhance TM affinity (Ye et al., 1994). In addition, these Arg residues in anion binding exosite 2 seemed to play a novel role in protein C activation. When protein C is activated by a complex of thrombin with TM lacking chondroitin sulfate (TM−CS), the activation rate rises as a function of Ca^{2+}, reaching an optimum at about 100 μM Ca^{2+} and then exhibits a sharp decline to about 10% the optimal rate at physiological Ca^{2+}. Comparison of kinetic parameters at the optimal and suboptimal Ca^{2+} levels indicates that the effect is on the K_m of the reaction (Kurosawa et al., 1987). Mutation of the Arg 93, 97, and 101 residues eliminates this high-affinity interaction with protein C (Ye et al., 1994).

In addition to the studies described above, other basic residues in anion binding exosite 2 appear to be important for binding interactions with heparin. These include Lys 240 (Church et al., 1989; Sheehan & Sadler, 1994) Arg 235 (Sheehan & Sadler, 1994) Arg 233 and Lys 236 (Sheehan & Sadler, 1994; Gan et al., 1994), and Arg 175 (Gan et al., 1994). All decrease heparin catalysis of antithrombin inhibition of thrombin.

In this study, we mutate Arg 93, 97, and 101 to Ala alone and in all combinations and investigate the effects of these mutations on (1) heparin catalysis of inhibition by antithrombin, (2) the ability of TM+CS to accelerate antithrombin inhibition, (3) formation of high-affinity protein C activation sites, and (4) the ability of the chondroitin sulfate to tighten the binding of TM to thrombin. The relative importance of the three Arg residues differs dramatically when assessed in these assays of anion binding exosite 2 function. Surprisingly, the Arg residues most responsible for heparin catalysis of thrombin inhibition differ from those required for chondroitin-dependent TM acceleration of the same reaction.

MATERIALS AND METHODS

Site-Directed Mutagenesis, Expression, and Purification of Human Thrombin Mutants. Construction of thrombin R93,97,101A has been previously described (Ye et al., 1994). Briefly, a unique *Nru*I restriction enzyme site was generated immediately next to the codon coding for Arg 101 in human prothrombin cDNA cloned into pUC (pUC-hII). This modified pUC-hII plasmid was then double-digested with *Bgl*II and *Nru*I restriction enzymes. This procedure released a 41 base pair DNA fragment which codes for the residues Tyr 89−Arg 101 of prothrombin. Twelve complementary oligonucleotides were synthesized that code for the missing residues in the *Bgl*II and *Nru*I gap with the exception that codons for Arg 93, Arg 97, and Arg 101 individually or in combination were substituted with an Ala. To express prethrombin 1 with these mutations, the *Bgl*II and *Eco*RI fragment of the expression vector, pNUT-PL2, producing prethrombin 1 (Rezaie, 1996) was replaced with the identical fragment of the modified pUC-prothrombin plasmid containing the desired substitution. The pNUT-PL2 mammalian expression vector contains a transferrin signal peptide for secretion, a *dhfr* gene for selection in methotrexate, and a 12-residue epitope for the Ca^{2+}-dependent monoclonal antibody HPC4 for purification (Rezaie, 1996). Baby hamster kidney (BHK) cells were transfected with this vector by Lipofectin (Gibco−BRL, Gaithersburg, MD) and methotrexate-resistant clones were selected and grown in 96-well plates. Supernatants were examined for expression by an ELISA using prethrombin 1 specific monoclonal antibody HPT 957 as catching antibody and HPC4 as detecting antibody. A high-expressing clone was identified and 20 L of cell culture supernatant was collected for each mutant, concentrated, and purified by HPC4 antibody linked to Affi-Gel 10 (Bio-Rad) as described (Ye et al., 1994). The prethrombin 1 mutant was activated with the prothrombinase complex and the mutant thrombin was purified by Mono S ion-exchange chromatography on FPLC.

Human thrombin (Miletich et al., 1978), human protein C (Esmon, 1993), bovine antithrombin (Owen, 1975), and recombinant soluble human TM (Liu et al., 1994) were prepared as described previously. Protein concentrations were determined using the following molecular weights and extinction coefficients ($E^{1\%}_{1cm}$) at 280 nm: human thrombin, 36 600 and 17.4 (Thompson et al., 1977); human protein C, 62 000 and 14.5 (Kisiel, 1979); and bovine antithrombin, 56 000 and 6.0 (Kurachi et al., 1976). The active-site concentration of recombinant thrombin derivatives was also determined as described (Mann et al., 1990) using BioCap-FPR-ck (biotinyl-ϵ-aminocaproyl-D-phenylalanylprolylarginine chloromethyl ketone) (Haematologic Technologies Inc.) as the active-site probe. The concentration of active enzyme determined by this method agreed within 10% of the values calculated on the basis of absorbance at 280 nm. Unfractionated heparin (porcine intestinal mucosa, sodium salt, grade II) was purchased from Sigma. Spectrozymes TH (SpTH) and PCa (SpPCa) were purchased from American Diagnostica, Greenwich, CT.

Thrombin Inhibition with Antithrombin. The rates of inactivation of human thrombin or the mutants were measured under pseudo-first-order rate conditions in 96-well vinyl plates at room temperature in 20 mM Tris-HCl (pH 7.5), 0.1 M NaCl, and 0.1% (w/v) gelatin. The wild-type or mutant thrombins were incubated with at least a 10-fold excess of antithrombin at room temperature for a period of time. SpTH was then added to give a final concentration of 0.2 mM. The rate of chromogenic substrate hydrolysis was measured with a Vmax kinetics microplate reader (Molecular Devices, Menlo Park, CA) and expressed as absorbance change at 405 nm (milli OD units per minute). The pseudo-first-order rate constant of inhibition was calculated by fitting the time-dependent change of the thrombin activity to a first-order rate equation using the ENZFITTER nonlinear regression program (R. J. Leatherbarrow, Elsevier, Biosoft):

$$A = A_0 e^{-k't} \quad (1)$$

where t is the time of the inhibition reaction, A is the residual thrombin activity at time t, A_0 is the initial thrombin activity at time zero, and k' is the pseudo-first-order rate constant.

The rate of inactivation of human thrombin in the presence of heparin was too fast to be measured under the above conditions. As an alternative, under pseudo-first-order rate conditions, the inhibition reaction was done in the presence of SpTH as a competing chromogenic substrate. The apparent pseudo-first-order rate constant of inhibition was estimated by fitting the absorbance at 405 nm vs time into

$$Ab = A_0/k_{app}(1 - e^{-k_{app}\,t}) + Ab_0 \quad (2)$$

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 zero, A_0 is the thrombin activity at time zero, 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

$$k' = k_{app}(1 + [S]/K_m) \quad (3)$$

where [S] is the concentration of the chromogenic substrate, SpTH, and K_m is the Michaelis−Menten constant of thrombin for SpTH.

Binding of Thrombin Mutants to Heparin−Agarose. Heparin−agarose was packed into a 2 mL FPLC column (HR5/5), and the column was subsequently connected to an FPLC system. The buffer used in pump A was 20 mM Tris-HCl (pH 7.5) and 0.1% (w/v) gelatin, and in pump B, 20 mM Tris-HCl (pH 7.5), 1.0 M NaCl, and 0.1% (w/v) gelatin. Two hundred microliters of either thrombin (200 nM) or thrombin R93,97,101A mutants (200 nM) was applied to the heparin column at 10% buffer B. The column was washed with 8.5 mL of this buffer before a 9 mL linear gradient from 10% to 100% B was applied. Fractions (200 μL) were collected and assayed for wild-type or mutant thrombin activity.

Protein C Activation. The initial rates of protein C activation by the wild-type and mutant thrombins were analyzed as a function of different Ca^{2+} concentrations as previously described (Galvin et al., 1987). Briefly, human plasma protein C (1 μM) was incubated with thrombin or the mutants (1 nM) in the presence of TM either lacking chondroitin sulfate (100 nM) or containing chondroitin sulfate (50 nM) and different concentration of Ca^{2+} (0−5 mM) in 20 mM Tris-HCl (pH 7.5) and 0.1 M NaCl (TBS) buffer, ionic strength 0.12, containing 1 mg/mL BSA for 10 min at room temperature. After inhibition of thrombin activity by addition of 200 μg/mL antithrombin, the initial rate of protein C activation was monitored by hydrolysis of 0.4 mM synthetic chromogenic substrate SpPCa in TBS buffer containing 1 mg/mL BSA. The rate of hydrolysis was measured at 405 nm at room temperature in a Vmax kinetic plate reader (Molecular Devices, Menlo Park, CA). The concentration of active protein C in reaction mixtures was determined by reference to a standard curve that was prepared by total activation of protein C at the time of each experiment. This was accomplished by total activation of 1 μM of each protein C with 10 nM thrombin in complex with 100 nM TM and 5 mM Ca^{2+} for 90 min at 37 °C. Under these experimental conditions, all protein C zymogen is completely activated in less than 30 min.

When protein C activation was used to determine the apparent dissociation constant between TM and thrombin or the thrombin mutants, the initial rates of protein C activation were determined as a function of different TM concentrations. The activation conditions were the same as those described above except that the initial rate of activation was monitored in the presence of varying concentrations of TM lacking or containing chondroitin sulfate (0−40 nM) in TBS buffer containing 5 mM Ca^{2+} and 1 mg/mL BSA, and the apparent dissociation constants were determined as described previously (Ye et al., 1994; Liu et al., 1994).

The Gibbs standard free energy (ΔG°_b) of complex formation was calculated from the estimated $K_{d(app)}$ values of thrombins and TM derivatives using the equation $\Delta G^\circ_b = -RT \ln K_{d(app)}$, where R is the gas constant and T is the absolute temperature. The changes in binding energy for each thrombin mutant relative to wild-type thrombin were calculated in the presence of both TM lacking chondroitin sulfate, $\Delta\Delta G^\circ_b$(TM−CS), and TM containing chondroitin sulfate, $\Delta\Delta G^\circ_b$(TM+CS). The changes in binding energy between TM lacking chondroitin sulfate and TM containing chondroitin sulfate, $\Delta\Delta G^\circ_b$[(TM−CS) − (TM+CS)], were also calculated for wild-type and mutant thrombins.

RESULTS

The thrombin Arg to Ala mutants were prepared as described under Materials and Methods. When chromatographed on a Mono S column at pH 6.0 and eluted with a linear gradient, elution of the mutants required 0.05−0.35 M lower NaCl concentrations than wild-type thrombin. The mutants all migrated as a single band with a molecular mass of 37 kDa on sodium dodecyl sulfate−polyacrylamide gel electrophoresis (data not shown).

Antithrombin inhibition of thrombin is accelerated by heparin and rabbit TM+CS (Bourin & Lindahl, 1993). As an initial step to investigate whether these processes required the same basic residues in anion binding exosite 2, we compared the influence of single, double, and triple Arg to Ala substitutions on the heparin optimum for inhibition and on the rate of inhibition at optimal heparin. In the absence of heparin, these mutations had very little effect on the rate of thrombin inhibition by antithrombin, with second-order rate constants varying from 0.63×10^4 to 0.99×10^4 M^{-1} s^{-1} (Table 1). Single mutations of any of these Arg residues increased the concentration of heparin required for maximum inhibition rates (Table 1) and decreased the rate of inactivation. Mutation of Arg 101 had the greatest effect on both parameters, with a 5-fold increase in the heparin concentration optimum and nearly a 10-fold decrease in inhibition rate. Arg 93 was slightly less important and Arg 97 had the least effect, especially with respect to heparin optimum. All double mutations resulted in a greater decrease in heparin-catalyzed thrombin inhibition than the single mutations. With the triple mutation, heparin nearly lost all of its catalysis power, as there was only a 2.4-fold increase in the inhibition rate constant and a 20-fold increase in the optimum heparin concentration (Table 1).

To determine if the changes in heparin catalysis were related to changes in heparin affinity, thrombin and the mutants were applied to a heparin−agarose column and eluted with a linear gradient (Figure 1). Arg 93 and Arg 101 mutation had the greatest effects on elution position, with the R97 mutation shifting the elution position only slightly. The elution position of the double mutants was shifted slightly further to the left than those of the single mutants but not as far as that of the triple R93,97,101A mutant. Thus, the mutations making the greatest impact on heparin-dependent thrombin inactivation by antithrombin are those that lead to the thrombin mutants most easily displaced from the heparin column.

TM+CS can also accelerate thrombin inhibition by antithrombin. In this case, the protein portion of TM allows

Table 1: Summary of Thrombin Inhibition Constants by Antithrombin[a]

thrombin	$k_2 \times 10^4$ M^{-1} s^{-1} without heparin	$k_2 \times 10^4$ M^{-1} s^{-1} with heparin	rate enhancement	optimal heparin concn (units/mL)
wild type	0.85 ± 0.23	648 ± 16.5	759	0.5
R93,97,101A	0.87 ± 0.27	2.1 ± 0.3	2.4	10.0
R93A	0.87 ± 0.23	126 ± 6.3	144	1.0
R97A	0.99 ± 0.27	208 ± 13.0	209	0.5−1.0
R101A	0.72 ± 0.09	56 ± 10.4	78	2.5
R93,97A	0.95 ± 0.27	18 ± 2.2	19	5.0
R93,101A	0.63 ± 0.18	7.4 ± 0.8	12	5.0
R97,101A	0.68 ± 0.24	12 ± 0.6	18	5.0

[a] Second-order association rate constants (k_2) for antithrombin inhibition of wild-type and R93,97,101A thrombins in the absence and presence of heparin are shown. The optimal concentration of heparin is indicated in the last column. All values are the average of at least three independent measurements with ± SE values.

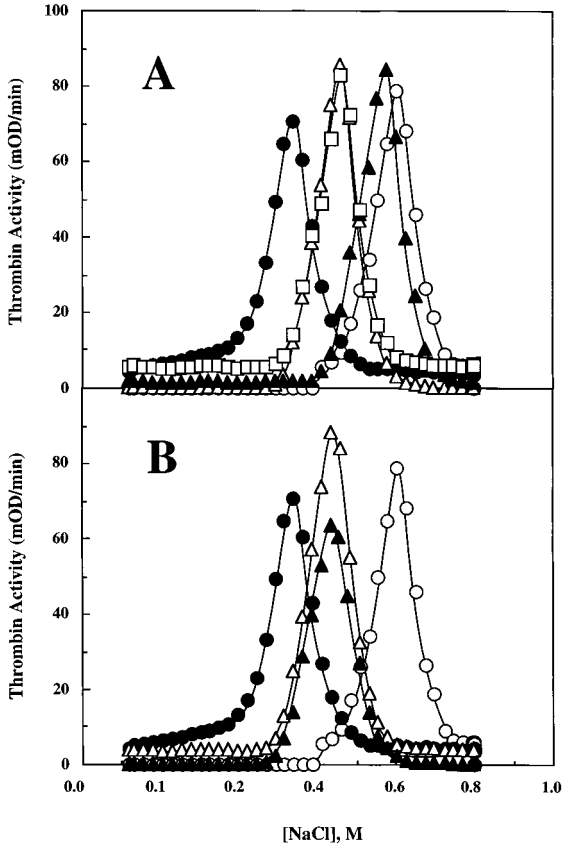

FIGURE 1: Binding of wild-type and mutant thrombins to a heparin−agarose. Thrombin and the mutants were applied to heparin agarose column as described under Materials and Methods. Following chromatography, the amidolytic activities of the eluted thrombin or the mutants toward Spectrozyme TH were plotted vs the NaCl concentrations. (**A**) (○) Wild-type thrombin, (●) R93,97,101A; (△) R93A mutant; (▲) R97A mutant; (□) R101A mutant. (**B**) (○) Wild-type thrombin; (●) R93,97,101A; (△) R93,97A; (▲) R97,101A. The expression yield of R93,101A was low and it was not analyzed by this method.

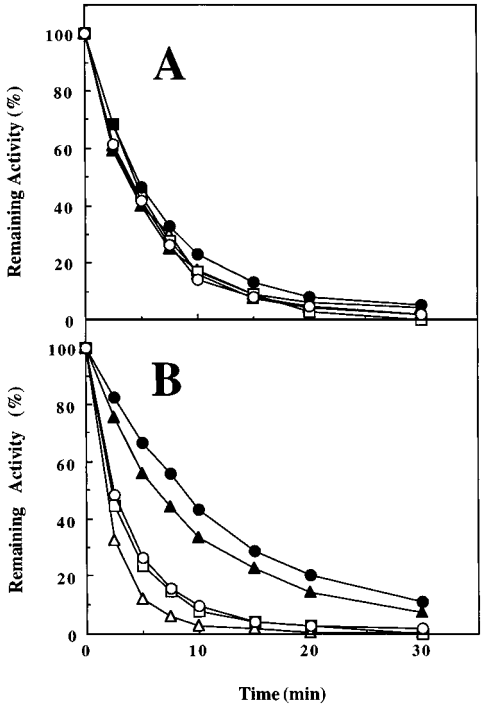

FIGURE 2: Effect of rabbit TM on inactivation of wild-type and mutant thrombins by antithrombin. Assays were performed as described under Materials and Methods in the absence (A) or the presence (B) of 50 nM rabbit thrombomodulin, with 2 nM wild-type or mutant thrombin, and 200 nM antithrombin. The remaining activities were measured at different reaction times. (○) Wild-type thrombin; (●) R93,97,101A mutant; (△) R93A mutant; (▲) R97A mutant; (□) R101A mutant.

interaction even when the glycosaminoglycan portion of the molecule is absent. Previous studies with Arg 93, 97, and 101 mutations had indicated that this region was probably involved in interacting with the chondroitin sulfate but did not address whether the chondroitin sulfate interaction with this region was important for enhancing inhibition by antithrombin. This region proved to be important for TM acceleration of the antithrombin reaction since TM+CS had almost no effect on the rate of inhibition of R93,97,101A mutant by antithrombin (Figure 2, Table 2). We then determined which of the Arg mutations was responsible for loss of this activity. In this case, mutation of Arg 97 was responsible for the loss in TM acceleration of the antithrombin reaction. As shown in Figure 2A, in the absence of TM, the mutations had minimal effect on thrombin inhibition by antithrombin. On the other hand, TM accelerated the antithrombin inhibition of wild-type, R93A, and R101A thrombins but not the inhibition of the R97A or R93,97,-101A mutants (Figure 2B). The mutation of Arg 93 was in fact associated with slight improvement in TM acceleration of the reaction (Figure 2B, Table 2). This result suggests that the charge neutralization of Arg 93, and even to a lesser extent Arg 101, improves the reactivity of antithrombin with the thrombin/TM+CS complex. Thus, the Arg residues within anion binding exosite 2 in thrombin differ in their contributions to heparin versus chondroitin sulfate-dependent catalysis of antithrombin inhibition.

Table 2: Summary of Thrombin Inhibition Constants by Antithrombin in the Absence and Presence of Rabbit Thrombomodulin[a]

thrombin	$k_2 \times 10^4$ M^{-1} s^{-1} without rabbit TM	$k_2 \times 10^4$ M^{-1} s^{-1} with rabbit TM	ratio
wild type	0.55 ± 0.02	2.85 ± 0.20	5.18
R93,97,101A	0.58 ± 0.02	0.60 ± 0.02	1.03
R93A	0.60 ± 0.01	4.68 ± 0.06	7.80
R97A	0.75 ± 0.01	1.01 ± 0.04	1.35
R101A	0.62 ± 0.01	2.90 ± 0.01	4.68

[a] Second-order association rate constants (k_2) for antithrombin inhibition of wild-type and R93,97,101A thrombins in the absence and presence of rabbit TM are shown. The ratio of the second-order association rate constants between the presence and absence of TM is indicated in the last column. All values are the average of at least three independent measurements with ± SE values.

To determine which residues were most responsible for enhancing chondroitin sulfate-dependent enhancement of TM affinity, the $K_{d(app)}$ for TM−CS and TM+CS was determined for each thrombin mutant. The mutations made less than a 2-fold change in $K_{d(app)}$ for TM−CS (Table 3). When TM+CS interaction with thrombin and the mutants was analyzed, much larger differences were seen between wild-type thrombin and the Arg → Ala mutants. Since there was some change in affinity with TM−CS, the relative contribution of each Arg to the CS dependence was expressed as a ratio of the affinity in the TM−CS to TM+CS. Arg 97 again made the greatest contribution to the binding affinity. To compare the contributions of the Arg residues to the binding, the $K_{d(app)}$ values were also converted to decrease in free energy of binding for both forms of TM (Table 3). With TM+CS, the difference in binding energy between wild-type thrombin and R93,97,101A thrombin was 6.1 kJ/mol. With TM−CS, this difference was only 1.6 kJ/mol. The single or double Arg mutants have decreased binding energy ranging from 0.6to 2.0 kJ/mol for TM lacking and from 1.1 to 4.8 kJ/mol for TM+CS (Table 3). These results indicate that the chondroitin sulfate enhances wild-type thrombin affinity for TM approximately 10-fold but enhances the affinity of R93,97,101A mutant only 1.6-fold and enhances the affinity of single or double Arg to Ala mutants 3−7-fold (Table 3).

Anion binding exosite 2 plays a role in the unusual Ca^{2+} dependence of protein C activation with TM−CS. Activation rates rise sharply with increasing Ca^{2+}, reaching an optimum, and then fall rapidly as Ca^{2+} concentrations increase into the physiological range (Figure 3A). The high activity observed at low Ca^{2+} has been observed previously with rabbit and human TM−CS. With rabbit TM, the high activity has been shown to be due to a decrease in the K_m

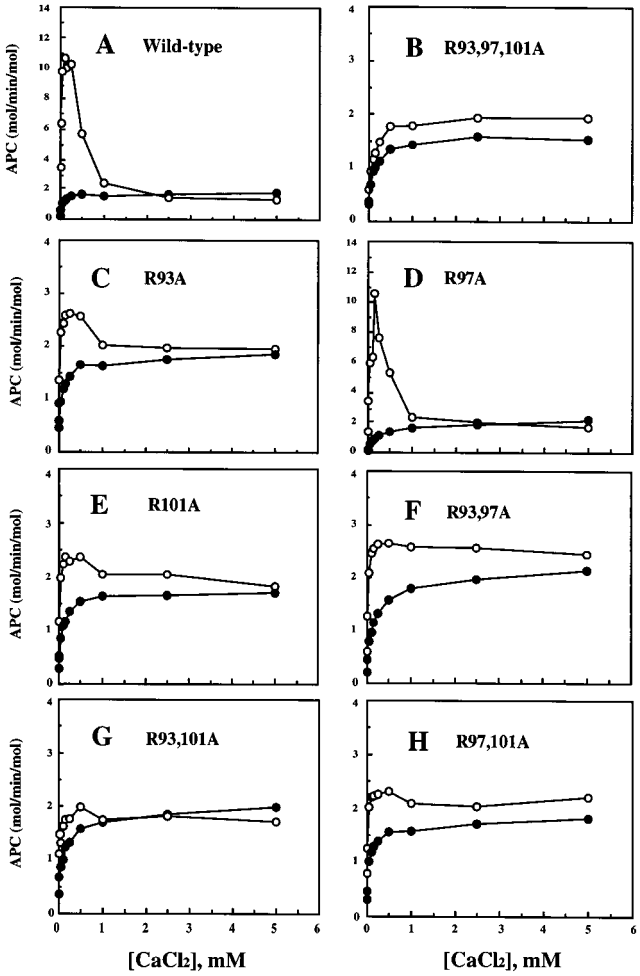

FIGURE 3: Ca^{2+} dependence of protein C activation by wild-type and mutant thrombins in complex with soluble human TM. The initial rates of protein C activation were analyzed at the indicated Ca^{2+} concentrations. The final concentrations of the reactants in the activation mixtures (30 μL) were as follows: human protein C (1 μM), thrombin or the thrombin mutant (2 nM), and TM containing and lacking chondroitin sulfate (50 and 100 nM, respectively). The rate of activated protein C generated was measured from the rate of hydrolysis of Spectrozyme PCa after inhibition of the thrombin activity by antithrombin. (**A**) Wild-type, (**B**) R93,97,101A, (**C**) R93A, (**D**) R97A, (**E**), R101A, (**F**), R93,-97A, (**G**) R93,101A, and (**H**) R97,101A thrombins as shown. (●) Thrombin/TM containing chondroitin sulfate: (○) thrombin/TM lacking chondroitin sulfate.

Table 3: Parameters of Thrombin Mutants for the Interaction with Thrombomodulin Lacking or Containing Chondroitin Sulfate[a]

thrombin	$K_{d(app)}$ (nM) TM−CS	$K_{d(app)}$ (nM) TM+CS	ratio (TM−CS)/(TM+CS)	$\Delta\Delta G°_b$(TM−CS) (kJ mol^{-1})	$\Delta\Delta G°_b$(TM+CS) (kJ mol^{-1})	$\Delta\Delta G°_b$[(TM−CS)−(TM+CS)] (kJ mol^{-1})
wild type	4.93 ± 0.19	0.51 ± 0.07	9.7			5.6
R93,97,101A	9.42 ± 0.46	5.90 ± 0.86	1.6	1.6	6.1	1.2
R93A	6.16 ± 1.48	0.81 ± 0.19	7.6	0.6	1.1	5.0
R97A	6.22 ± 0.12	1.49 ± 0.27	4.2	0.6	2.7	3.5
R101A	10.50 ± 0.70	1.64 ± 0.14	6.4	1.9	2.9	4.6
R93,97A	11.05 ± 1.22	3.58 ± 0.39	3.1	2.0	4.8	2.8
R93,101A	8.23 ± 0.53	1.27 ± 0.21	6.5	1.3	2.3	4.6
R97,101A	8.47 ± 0.94	1.91 ± 0.21	4.4	1.3	3.3	3.7

[a] Apparent dissociation constants [$K_{d(app)}$] of TM containing or lacking chondroitin sulfate with wild-type and mutant thrombins are shown. All values are the average of at least three independent measurements with ± SE values. Values of $\Delta G°_b$ were calculated using the relationship $\Delta G°_b = -RT \ln (K_{d(app)})$. The values of $\Delta\Delta G°_b$(TM−CS) and $\Delta\Delta G°_b$(TM+CS) represent the decrease in binding energy caused by a particular mutation relative to wild-type thrombin in the presence of TM lacking or containing chondroitin sulfate. The values of $\Delta\Delta G°_b$[(TM−CS)−(TM+CS)] represent the decrease in binding energy between two forms of TM for all thrombin derivatives.

for protein C (Kurosawa et al., 1987). We confirmed this observation with human TM−CS (data not shown). With TM+CS, this site appears to be masked and protein C activation exhibits a simple hyperbolic Ca^{2+} dependence (Figure 3A). For simplicity of discussion, the activity at low Ca^{2+} concentration will be referred to as high-affinity protein C interaction. This high-affinity protein C interaction is not detected with R93,97,101A thrombin (Ye et al., 1994). To identify the Arg residues that participate in the high-affinity protein C interaction, we examined the Ca^{2+} dependence of protein C activation with each of the single Arg to Ala mutations (Figure 3). The high-affinity interaction was maintained in the Arg 97 thrombin mutant but almost completely lost in either the Arg 101 or Arg 93 mutants. These results suggest that Arg 101 and Arg 93 contribute to the high-affinity interaction with protein C.

The high-affinity interaction with protein C is dependent on the presence of the Gla- domain (Kurosawa et al., 1987; Ye et al., 1994). To determine if the interaction was specific to the Gla domain of protein C, we prepared a chimera in which the Gla domain of protein C was replaced with the corresponding region of prothrombin (PC-PT Gla).[2] With TM−CS and wild-type thrombin, the chimera retained most of its high-affinity interaction as evidenced by the much higher rates of activation at low vs high Ca^{2+} concentrations (compare Figure 3A for protein C and Figure 4A for the chimera). When the Gla domain was not completely carboxylated during biosynthesis, most of the high-affinity interaction was lost (Figure 4A, △). With TM+CS and wild-type thrombin, a simple hyperbolic Ca^{2+} dependence of chimera activation was observed similar to that seen with protein C. The Ca^{2+} dependence of PC-PT Gla chimera and protein C activation by wild-type thrombin−TM+CS complex were similar (Figure 4A). With thrombin R93,97,101A, the chimera exhibited a simple hyperbolic Ca^{2+} dependence that was independent of the chondroitin sulfate moiety (Figure 4B). As was the case with protein C, Arg residues 93 (Figure 4C) and 101 (Figure 4E) appeared to play the major role in the high-affinity interaction with the chimera since mutation of either of these residues resulted in diminished activation rates at low Ca^{2+} and little effect at high Ca^{2+}. Arg 97 appeared to play little role in interaction of thrombin with either the chimera (Figure 4D) or protein C (Figure 3D). The high-affinity interaction site at low Ca^{2+} concentration for the chimera was also lost with all double Arg → Ala mutants (shown only for R93,97A thrombin in Figure 4F).

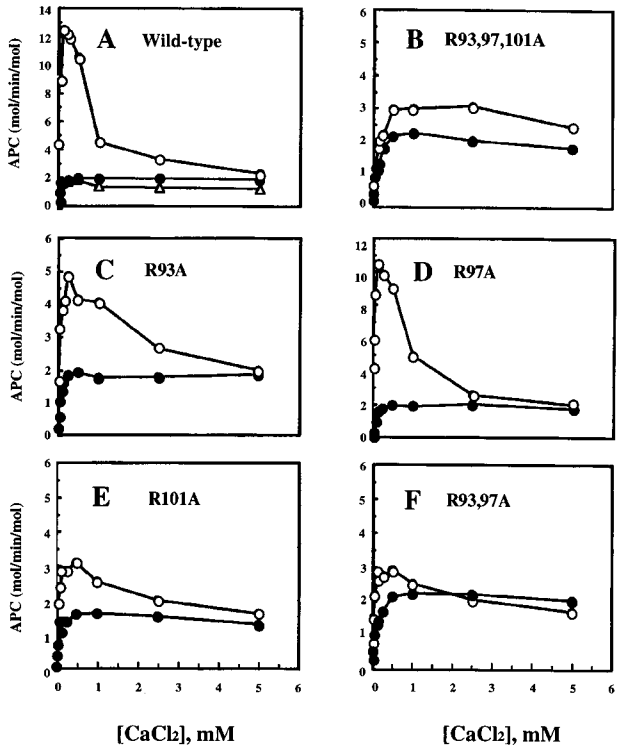

FIGURE 4: Ca^{2+} dependence of PC-PT Gla activation by wild-type and mutant thrombins in complex with soluble human TM. The initial rates of protein C activation were analyzed at the Ca^{2+} concentrations indicated. The final concentrations of the reactants in the activation mixtures (30 μL) were as follows: PC-PT Gla (1 μM), thrombin or the thrombin mutant (2 nM), and TM containing and lacking chondroitin sulfate (50 and 100 nM, respectively). Protein C activation rates were measured as described under Materials and Methods. (●) PC-PT Gla activation by thrombin/TM containing chondroitin sulfate; (○) PC-PT Gla activation by thrombin/TM lacking chondroitin sulfate; (△) undercarboxylated PC-PT Gla activation by thrombin/TM lacking chondroitin sulfate (with wild-type thrombin only in panel A). (**A**) Wild-type, (**B**) R93,-97,101A, (**C**) R93A, (**D**) R97A, (**E**) R101A, and (**F**) R93,97A thrombins are shown.

DISCUSSION

Our results indicate that the functions of anion binding exosite 2 are mediated by different basic residues. Arg 93 and Arg 101 were the major contributors to the heparin acceleration of thrombin inhibition by antithrombin and were required for high-affinity protein C interaction by activation complexes involving TM−CS. In contrast, these residues played little apparent role in the TM-dependent acceleration of thrombin inhibition by antithrombin, a reaction that is dependent on the chondroitin sulfate moiety of TM. In this case, the reaction was dependent on the presence of Arg 97. Similarly, Arg 97 played the most important role in chondroitin sulfate-dependent tightening of the thrombin/TM complex. Thus, there is considerable specificity to the functions of this exosite and it is not simply functioning as a cationic region that interacts relatively nonspecifically with anionic polymers.

Since TM will interact with thrombin in a chondroitin sulfate-independent manner, before the present studies were completed, it was unclear as to whether chondroitin sulfate interaction with thrombin would play any role in the ability to accelerate antithrombin inhibition. It is now clear, however, that mutations in anion binding exosite 2, particularly Arg 97, do have a major impact on the reactivity with antithrombin. It is unlikely that this mutation is perturbing antithrombin interaction nonspecifically since the reactivity in the absence of heparin was essentially unaltered. Furthermore, of the three Arg residues examined, Arg 97 mutation had the least influence on heparin affinity or function.

The very unusual Ca^{2+} dependence of protein C activation depends on the Gla domain and is characterized by a high-affinity substrate interaction (low K_m) with TM−CS at low Ca^{2+} concentrations. The present study indicates that this phenomenon is not protein C Gla domain-specific since substitution of the protein C Gla domain with the prothrom-

[2] M. D. Smirnov, A. R. Rezaie, C. T. Esmon, and N. L. Esmon, unpublished results.

bin counterpart leaves the phenomenon largely unperturbed. The interaction does require carboxylation, however, since undercarboxylated forms of this substrate do not exhibit the characteristic high-affinity interaction. This high-affinity interaction with protein C is also eliminated when TM contains chondroitin sulfate. The simplest interpretation of this phenomenon would be that the chondroitin sulfate binds directly to residues that contribute to the interaction. Single mutations indicate that Arg 101 and Arg 93 are critical for this substrate interaction but play less of a role in modulating TM affinity for thrombin. Mutations of these residues does decrease TM+CS affinity slightly, suggesting that the chondroitin sulfate might bind to or near these residues and that binding could block this protein C activation activity. Protein C binding to thrombin (modified to block the active site) or the thrombin/TM complex has been observed in sedimentation equilibrium experiments. Increases in Ca^{2+} were observed to decrease protein C affinity of thrombin or the thrombin/TM complex (Olsen et al., 1992). In these experiments, a proteolytic derivative of rabbit TM was used that has functional properties similar to the TM−CS used in these studies. At present, however, there are no data demonstrating that high-affinity interaction involves direct protein C interaction with anion binding exosite 2. Therefore, these functional changes could be mediated by conformational changes that result from charge neutralization on this surface.

Arg 93 and Arg 101 are located close together (4 Å) in the crystal structure of thrombin, and they are separated about 12 Å from Arg 97. Arg 97 is positioned very near the 60 loop that has been implicated as a major factor in determining thrombin specificity (Bode et al., 1992; Le Bonniec et al., 1993; Tulinsky & Qiu, 1993; Rezaie, 1996; Rezaie & Olson, 1997). Substrates like fibrinogen exit the catalytic center of thrombin very near Arg 97, and Arg 97 makes contacts with fibrinopeptide A in the crystal structure (Bode et al., 1992). It is possible that the interaction of Arg 97 with chondroitin sulfate predicted from these studies directs the chondroitin sulfate in the general direction of the substrates and contributes to the observed enhancement by chondroitin sulfate to TM-mediated inhibition of thrombin's fibrinogen clotting activity.

Finally, substitution of Arg 93 of thrombin with Glu (R93E) in a previous study was shown to eliminate most of the effector function of heparin in acceleration of thrombin inhibition (Sheehan & Sadler, 1994). This result appeared to suggest that most of the binding energy of thrombin/heparin complex formation arises from interaction of heparin with Arg 93. In this study, on the other hand, heparin still accelerated antithrombin inhibition of R93A 144-fold. The difference between heparin acceleration of R93E and R93A thrombins suggests that repulsive interactions of heparin with the negatively charged Glu93 contributed to a dramatic decrease in the cofactor function of heparin observed in the earlier study. A dramatic decrease in the effector function of heparin was noticed only when all three Arg 93, Arg 97, and Arg 101 were mutated to Ala. These results are consistent with the finding of Olson et al. that 5−6 ionic interactions are required for the effective binding of heparin on thrombin either in a binary complex or in the ternary complex with antithrombin (Olson et al., 1991; Olson & Björk, 1991). Furthermore, in another mutagenesis study, Gan et al. (1994) proposed a critical role for the basic residues R175, R233, K236, and K240 in heparin catalysis of thrombin inhibition by antithrombin. Taken together, therefore, these results suggest that the basic residues may act cooperatively to align heparin on the exosite 2 of thrombin and that optimal interaction requires the presence of a certain minimum number of these basic residues.

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