# Contribution of Lysine 60f to S1′ Specificity of Thrombin†

Alireza R. Rezaie*,‡ and Steven T. Olson§

*Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104, and Center for Molecular Biology of Oral Diseases, University of Illinois−Chicago, Chicago, Illinois 60612*

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ABSTRACT: Lys60f has been proposed to limit the S1′ substrate binding site specificity of thrombin to small polar P1′ residues by occluding the S1′ binding pocket, based on the X-ray crystal structure of thrombin. To test this proposal, we prepared a Lys → Ala (K60fA) mutant of recombinant thrombin and determined whether this mutation enhanced the reactivity of thrombin with a variant inhibitor [antithrombin (AT)-Denver] and a substrate (protein C) containing poorly recognized P1′ Leu residues. AT-Denver in the presence of heparin inhibited K60fA thrombin with a second-order association rate constant [$k = (4.2 \pm 0.1) \times 10^5$ M$^{-1}$ s$^{-1}$] that was 3.2-fold faster than thrombin [$k = (1.3 \pm 0.1) \times 10^5$ M$^{-1}$ s$^{-1}$]. Wild-type AT (P1′ Ser) under the same conditions inhibited K60fA thrombin with a 2.5-fold slower rate constant [$k = (1.1 \pm 0.1) \times 10^7$ M$^{-1}$ s$^{-1}$] than thrombin [$k = (2.8 \pm 0.1) \times 10^7$ M$^{-1}$ s$^{-1}$]. These results indicate an overall 8.3-fold improvement in the recognition of the P1′ Leu of AT-Denver by K60fA thrombin over that of wild-type thrombin; i.e., the K60fA mutation partly overcomes the defect in thrombin inhibition produced by the P1′ mutation in AT-Denver. Resolution of the two-step reactions of AT and AT-Denver with wild-type and mutant thrombins revealed that the enhanced recognition of P1′ Leu in AT-Denver by K60fA thrombin occurs primarily in the second reaction step in which a noncovalent AT−thrombin encounter complex is converted to a stable, covalent complex. Thrombin K60fA activated Gla-domainless protein C (GDPC) ∼2- and ∼4-fold faster than thrombin in the presence and absence of thrombomodulin (TM), respectively, consistent with an improved interaction of the Leu P1′ residue with the mutant S1′ pocket. In contrast, the mutant thrombin clotted fibrinogen (P1′ Gly) ∼3-fold slower than thrombin. Kinetic analysis revealed that the improvement in the catalytic rate of activation of GDPC by K60fA thrombin in the presence of TM was localized in the second reaction step, as reflected by an ∼2-fold increase in $k_{cat}$. Direct binding studies showed that the K60fA mutation minimally affected the affinity of thrombin for $Na^+$, indicating that the changes in S1′ site-specificity of K60fA thrombin did not result from altering the allosteric transition induced by $Na^+$. We conclude that Lys60f limits the P1′ substrate and inhibitor specificity of thrombin by influencing the size and polarity of the S1′ site which thereby affects the stability of the transition state for cleavage of the scissile bond in the second reaction step.

Thrombin is a multifunctional serine protease with a high degree of substrate and inhibitor specificity. A key factor which accounts for the restricted specificity of thrombin and other coagulation proteases is the specific interactions of S and S′ subsite residues in the extended binding pocket of these enzymes with residues at the P and P′ sites of their substrates and inhibitors.[1] Similar to trypsin, thrombin and other coagulation proteases show specificity toward substrates and inhibitors containing the basic residues, Arg and Lys, at the P1 positions due to interaction with Asp189 in the S1 pockets of these enzymes. The importance of the P1 residue for enzyme specificity is demonstrated in the natural variant $\alpha_1$-antitrypsin Pittsburgh, in which the substitution of the P1 residue Met with Arg results in more than a 1000-fold increase in the rate of thrombin and activated protein C (APC)[2] inhibition by the variant serpin (Heeb et al., 1990). In other studies, mutagenesis of P2 or P3 residues in several serpin inhibitors (Hopkins et al., 1995; Cooper & Church, 1995) or substrates (Rezaie & Esmon, 1992, 1994) has resulted in mutants which reacted with thrombin with altered specificities. The recent X-ray crystal structure determination of thrombin has provided further clues to the molecular basis of restricted substrate and inhibitor specificity of thrombin. The active site groove of thrombin is surrounded on both sides by two insertion loops, B and C, that are not conserved in other serine proteases (Stubbs & Bode, 1993). Molecular modeling (Bode et al., 1989, 1992) and mutagenesis studies (Le Bonniec et al., 1992, 1993) suggest that both of these loops restrict the catalytic pocket of thrombin for access by

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* Address correspondence to this author at the Oklahoma Medical Research Foundation, Cardiovascular Biology Research, 825 N.E. 13th St., Oklahoma City, OK 73104. Phone: (405) 271-7264. Fax: (405) 271-7417.

‡ Oklahoma Medical Research Foundation.

§ University of Illinois−Chicago.

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[1] Nomenclature of Schechter and Berger (1967) used to describe the subsites of interaction between a protease and its substrate. Amino acid residues of the substrate are referred to as P1, P2, etc. on the N-terminal side of the substrate scissile bond and those on the C-terminal side as P1′, P2′, etc. The corresponding sites on the enzyme where substrate residues interact are designed S1, S2, etc. and S1′, S2′, etc., respectively.

[2] Abbreviations: AT, antithrombin; thrombin K60fA, a thrombin mutant in which Lys residue 60f in the chymotrypsin numbering system (Bode et al., 1989) is converted to Ala; TM, thrombomodulin; GDPC, Gla-domainless protein C, protein C from which residues 1−45 were removed by the recombinant DNA methods; APC, activated protein C; PEG, poly(ethylene glycol).

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the Kunitz inhibitors, bovine pancreatic trypsin inhibitor and tissue factor pathway inhibitor. In contrast to the Kunitz inhibitors, the insertion loops have been shown to play a positive role for optimal interaction of thrombin with macromolecular substrates and inhibitors. Deletion of either one of these loops resulted in mutant thrombins that bound to Kunitz inhibitors with higher affinities, but the mutants activated the natural substrates, protein C and fibrinogen, at slower rates and were inhibited by the serpin, antithrombin, poorly (Guinto et al., 1994; Le Bonniec et al., 1992, 1993). In addition to the insertion loops, thrombin has two patches of positively charged residues on its surface, and binding of cofactors (Esmon, 1989), substrates (Jakubowski & Owen, 1989), and inhibitors (Rydel et al., 1990) on these sites modulates thrombin specificity by allosteric mechanisms.

In contrast to S-subsite specificity, less information is available about the S′-subsite specificity of thrombin which interacts with residues at the C-terminal side of the cleavage site. The X-ray crystal structure of thrombin in complex with small inhibitors indicates that the S1′ site of thrombin is occluded by Lys60f, restricting the specificity of this site to small residues with polar side chains (Bode et al., 1989). A frequent occurrence of small residues at the P1′ positions of natural inhibitors and protein substrates of thrombin is consistent with the structural data. Characterization of the reactivity of thrombin with natural and engineered variants of antithrombin in which Ser394 at the P1′ position is substituted with other amino acids has experimentally confirmed the specificity of the S1′ site for small polar P1′ residues (Stephen et al., 1988; Theunissen et al., 1993). Detailed kinetic analysis of the reaction of thrombin with the natural Ser → Leu P1′ variant antithrombin-Denver indicated that the defective function of the mutant inhibitor was localized in the second of two reaction steps in which an initial noncovalent enzyme−inhibitor complex is converted to a stable, covalent complex (Olson et al., 1995). Such results indicated that the S1′−P1′ interaction contributed to thrombin specificity by stabilizing the transition state for attack of the catalytic serine on the inhibitor scissile bond prior to the trapping of enzyme at the tetrahedral or acyl-intermediate stage of a regular substrate reaction (Olson et al., 1995).

To test the hypothesis that Lys60f restricts the P1′ substrate and inhibitor specificity of thrombin, we have prepared a mutant of prethrombin 1 in which Lys60f is substituted with Ala (K60fA). The K60fA mutant was expressed in mammalian cells, and, following purification and activation, its enzymatic properties were characterized with respect to inhibition by wild-type and Denver mutant antithrombins, activation of protein C, and fibrinogen clotting. We demonstrate that the thrombin K60fA mutation significantly improves the rate at which antithrombin-Denver inhibits thrombin. Resolution of the two-step enzyme−inhibitor reaction indicates that the improvement in the reactivity of thrombin K60fA with the Denver mutant is localized in the second reaction step. Similarly, thrombin K60fA cleaves the Arg−Leu activation bond of GDPC[2] ~4-fold faster than thrombin whereas the Arg−Gly activation bonds of fibrinogen are cleaved slower based on the ~3-fold slower rate of fibrinogen clotting measured under conditions where bond cleavage is rate-limiting. As with the antithrombin-Denver reaction, the improvement in the rate of protein C activation was found to be due to an increase in the $k_{cat}$ with no change in the $K_M$. The altered reactivity of the K60fA thrombin with substrates and inhibitors did not result from effects on the $Na^+$-induced allosteric transition of thrombin (Dang et al., 1995) since $Na^+$ binding was minimally affected by the K60fA mutation. These results indicate that Lys60f is a critical determinant of the P1′ specificity of thrombin's reaction with the natural substrates, protein C and fibrinogen, and the serpin inhibitor, antithrombin. The results further suggest that the S1′−P1′ interaction in thrombin primarily contributes to transition-state stabilization in both reactions, similar to other serine proteases (Fersht et al., 1973).

## MATERIALS AND METHODS

*Expression and Purification of Recombinant and Plasma Proteins.* Antithrombin and antithrombin-Denver were purified from normal and patient plasma, as previously described (Olson et al., 1995). Recombinant Gla-domainless protein C (GDPC) and TM4-6 fragment were expressed in the RSV-PL4 expression system and purified by immunoaffinity chromatography using the HPC4 monoclonal antibody linked to Affigel-10 (Bio-Rad), as previously described (Rezaie & Esmon, 1992). Wild-type and the K60fA mutant of prethrombin 1 were expressed in the pNUT-PL2 expression system and similarly purified by immunoaffinity chromatography using the HPC4 monoclonal antibody linked to Affigel-10, as previously described (Rezaie, 1996). Site-directed mutagenesis was performed by polymerase chain reaction methods, as previously described (Rezaie & Esmon, 1992; Higuchi et al., 1988). Prethrombin 1 was activated by the prothrombinase complex, and thrombin was purified on an FPLC Mono S column (Pharmacia), as previously described (Ye et al., 1994). Plasma-derived thrombin was a generous gift of John Fenton of the New York State Department of Health.

The concentrations of recombinant wild-type thrombin and K60fA thrombin as well as plasma thrombin were determined from the absorbance at 280 nm, assuming a molecular weight of 36 600 and an extinction coefficients ($E^{1\%}_{1cm}$) of 17.1. The concentration of active enzymes was also determined by the active-site specific immunoassay (Mann et al., 1990) using BioCap-FPR-CK (biotinyl-$\epsilon$-aminocaproyl-D-phenylalanineprolylarginine chloromethyl ketone) (Haematologic Technologies Inc., VT) as a probe or by active-site titration with methylumbelliferyl guanidinobenzoate (Olson et al., 1995). Active-site concentrations agreed within 10% of the values calculated based on the absorbance at 280 nm. Titrations of wild-type and mutant thrombins with wild-type antithrombin yielded stoichiometries of inhibition of 1.0−1.1 mol of inhibitor/mol of enzyme, confirming the active-site titration data. Recombinant wild-type and plasma thrombins were used interchangeably based on their indistinguishable properties (Ye et al., 1994). All proteins were homogeneous as determined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

*Synthetic Substrate Cleavage.* $K_M$ and $k_{cat}$ values for cleavage of the fluorescent substrate tosyl-GPR-7-amido-4-methylcoumarin (Sigma) by wild-type and K60fA thrombins were determined by measuring the initial rate of substrate cleavage by 10 pM thrombin as a function of substrate concentrations ranging from 0.5 to 20 $\mu$M. Buffer, temperature, and fluorometer settings for monitoring the fluorescent product of cleavage were as given for the inhibition kinetic

experiments described below. The fluorometer was standardized with 7-amido-4-methylcoumarin to provide a full-scale reading of 0.1 $\mu$M fluorescent product. The dependence of initial rates of substrate cleavage on substrate concentration was fit by nonlinear regression to the Michaelis−Menten equation to yield $K_M$ and $k_{cat}$. $K_M$ and $k_{cat}$ values for cleavage of the chromogenic substrate S2266 (Kabi Pharmacia/Chromogenix, Franklin, OH) by wild-type and K60fA thrombins were determined by measuring the initial rate of substrate cleavage by 5 nM thrombin as a function of substrate concentrations ranging from 30 to 1000 $\mu$M. The rate of *p*-nitroaniline release was measured at 405 nm at room temperature by a Vmax kinetic plate reader (Molecular Devices, Menlo Park, CA). The buffer was 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.5 (TBS), containing 0.1% PEG 8000. Kinetic constants for cleavage of the chromogenic substrate, S2238, by wild-type and mutant thrombin were determined in 20 mM sodium phosphate, 0.1 M NaCl, 0.1 mM EDTA, and 0.1% PEG 8000, pH 7.4 at 25 °C, by analysis of progress curves for complete hydrolysis of substrate. Substrate concentrations ranged from 4 to 17 $\mu$M, and enzyme concentrations were 0.5−0.8 nM. Eight to ten progress curves for each thrombin were computer-fit by nonlinear regression to the integrated Michaelis−Menten equation. The apparent $K_M$ values obtained by this analysis were dependent on the substrate concentration, indicating the existence of product inhibition, in agreement with previous reports (Lottenberg et al., 1983). Secondary plots of apparent $K_M$ values against substrate concentration were linear and provided the true $K_M$ as well as the product inhibition constant, $K_I$. True $k_{cat}$ values were obtained by suitable corrections for product inhibition of the substrate concentration-independent apparent $k_{cat}$ value (Segal, 1975). Measured $K_I$ values were 6−8-fold higher than $K_M$ values and indistinguishable within experimental error for the two forms of thrombin.

*Inhibition Kinetics.* The second-order inhibition rate constants for the heparin-catalyzed inhibition of wild-type and mutant thrombins by antithrombin and antithrombin-Denver were determined as described previously (Olson et al., 1995). The buffer was 20 mM sodium phosphate, 0.1 M NaCl, 0.1 mM EDTA, and 0.1% PEG 8000 plus 1 mg/mL bovine serum albumin (BSA) with an ionic strength of 0.15 M in pH 7.4 at 25 °C. Reactions contained 200 nM antithrombin or 100 nM antithrombin-Denver, 1 nM wild-type or mutant thrombin, and high-affinity heparin ($M_r$ ~8000) concentrations ranging from 0.25 to 10 nM in 50 $\mu$L. Identical samples were quenched after different reaction times with 0.95 mL of 50 $\mu$M tosyl-Gly-Pro-Arg-7-amido-4-methylcoumarin (Sigma) containing 50 $\mu$g/mL polybrene. The residual thrombin activity was then measured from the linear rate of hydrolysis of substrate monitored fluorometrically with excitation at 380 nm and emission at 440 nm. The loss in thrombin activity was fit by a single-exponential decay process which yielded the pseudo-first-order rate constant, $k_{obs}$. Second-order rate constants for uncatalyzed and heparin-catalyzed reactions were obtained from the intercept and slope, respectively, of the linear dependence of $k_{obs}$ on heparin concentration (Björk et al., 1992). Correction for the extent of heparin saturation of antithrombin was done by dividing the slopes by the factor $[AT]_o/(K_{AT,H} + [AT]_o)$ where $[AT]_o$ is the total antithrombin concentration and $K_{AT,H}$ is the dissociation constant for the antithrombin−heparin interaction, measured to be 15 nM for both wild-type and variant antithrombins. Reported errors in rate constants represent ±2 SE.

To resolve the two reaction steps of antithrombin and antithrombin-Denver reactions with wild-type and K60fA thrombins, $k_{obs}$ was measured as a function of antithrombin−heparin complex concentration. Antithrombin-Denver reactions were done as above except inhibitor concentrations ranged from 100 to 500 nM, high-affinity heparin concentrations were varied from 20 to 400 nM, and the thrombin concentration was 1 nM. Wild-type antithrombin reactions were done by continuous inhibition assays in a stopped-flow fluorometer (Applied Photophysics) using the fluorogenic substrate as a reporter, similar to the method previously described (Gan et al., 1994). In this case, antithrombin concentrations ranged from 100 to 2250 nM, high-affinity heparin concentrations from 25 to 1500 nM, thrombin from 1 to 30 nM, and the substrate was present at 5 $\mu$M. The concentrations of antithrombin−heparin complexes were calculated using the measured $K_D$ of 15 nM for the interaction. The dependence of $k_{obs}$ on [antithrombin−heparin complex] was computer-fit by a rectangular hyperbola to provide the $K_D$ for the formation of a ternary heparin−antithrombin−thrombin encounter complex and a limiting first-order rate constant for conversion of this encounter complex to a stable complex (Olson et al., 1995). For continuous inhibition assays, $k_{obs}$ was plotted vs the effective antithrombin−heparin complex concentration given by the ratio $[AT-heparin]/(1 + [S]_o/K_M)$, where $[S_o]$ represents the concentration of fluorogenic substrate and $K_M$ is the Michaelis constant for thrombin cleavage of this substrate, to correct for competition by the substrate (Olson et al., 1993).

*Protein C Activation.* The initial rates of Gla-domainless protein C activation by thrombin and K60fA thrombin were measured in TBS containing 1 mg/mL BSA and either 2.5 mM $Ca^{2+}$ or 1 mM EDTA. In the presence of $Ca^{2+}$, the rates were also measured in the presence of thrombomodulin fragment 4-6 (TM4-6). In the presence of TM4-6 and $Ca^{2+}$, the reactions were done with 2 $\mu$M GDPC and 1 nM thrombin or K60fA thrombin for 15 min at room temperature. In the presence of 1 mM EDTA, the reaction conditions were the same except that the concentration of thrombin was increased to 20 nM. In the presence of $Ca^{2+}$, but no TM, the concentration of thrombin was 100 nM, and the time course of activation was increased to up to 60 min. After varying times, samples of reactions were transferred to TBS buffer containing 200 $\mu$g/mL antithrombin to inhibit thrombin activity. At this concentration of antithrombin, the activity of thrombin was rapidly inhibited, while the amidolytic activity of active GDPC remained stable for more than 30 min. The amidolytic activity of active GDPC in the activation reactions was monitored by hydrolysis of 400 $\mu$M Spectrozyme PCa (American Diagnostica Inc., Greenwich, CT) 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 derivative in reaction mixtures was determined by reference to a standard curve which was prepared by total activation of GDPC at the time of each experiment. This was accomplished by total activation of 1 $\mu$M of each protein C derivative with 10 nM thrombin in complex with 100 nM TM4-6 and 2.5 mM $Ca^{2+}$

for 90 min at 37 °C. Under these experimental conditions, all protein C zymogen was completely activated in less than 30 min. When a detailed kinetic analysis was done to determine values for $K_M$ and $k_{cat}$ of protein C activation, the initial rates of activation were measured as a function of GDPC concentration with 1 nM thrombin in complex with 100 nM TM4-6, as described above and, in detail, previously (Rezaie & Esmon, 1992). Fixed reaction times of 5 min were employed for each GDPC concentration. Over this time, only the initial rate of activation was measured, and less than 1% substrate was activated.

*Clotting Assays.* The activities of thrombin and thrombin K60fA were compared in clotting assays using human plasma or purified fibrinogen (Kabi Diagnostica, Stockholm, Sweden). Clotting assays with pure fibrinogen were done at fibrinogen concentrations well below the $K_M$ for cleavage of this substrate by thrombin. Under such conditions, the clotting time is limited by the rate of cleavage of fibrinogen by thrombin (De Cristofro & Di Cera, 1991). Clotting was initiated by adding 1−10 nM wild-type or K60fA thrombin to a solution of 0.25 $\mu$M fibrinogen in 0.1 M Hepes, 0.1 M NaCl, 10 mM $CaCl_2$, and 0.1% PEG 8000, pH 7.4 at 25 °C. Clot formation was continuously monitored from the sigmoidal increase in absorbance at 350 nm due to light scattering until a plateau absorbance was reached. The clotting time was determined as the time corresponding to the intersection of a line drawn through the steepest part of the clotting curve with the base line absorbance (De Cristofro & Di Cera, 1991). At least three concentrations of thrombin were tested, each in duplicate, with less than 3% variation in duplicates. Human plasma clotting was initiated by addition of 100 $\mu$L of 2 units/mL thrombin (∼22 nM) to 100 $\mu$L of citrated human plasma at 37 °C, to give a final concentration of 1 unit/mL thrombin, and the clotting time was measured with an ST4 Biocoagulometer (Diagnostica/Stago, Asnieres, France). Under these conditions, a clotting time of ∼18 s was obtained for wild-type thrombin. The same conditions were used for fibrinogen clotting except that 100 $\mu$L of 6 mg/mL human fibrinogen in 0.15 M NaCl, 0.02 M Tris-HCl, pH 7.5, was used instead of human plasma. The clotting assays were done with concentrations ranging from 0.125 to 6 units/mL thrombin (∼1.4−66 nM). log−log plots of clotting time vs thrombin concentration were linear with indistinguishable slopes for the two thrombins in all clotting assays.

*$Na^+$ Binding Titrations.* Binding of $Na^+$ to wild-type and K60fA thrombins was measured at pH 8.0, ionic strength 0.18, and 25 °C by titrating the intrinsic protein fluorescence enhancement which accompanies this binding as described by Wells and Di Cera (1992). Briefly, thrombins were dialyzed into 5 mM Tris-HCl, 0.1% PEG 8000, pH 8.0, buffer and then diluted 10-fold to equivalent concentrations of 100 nM in buffer containing either 0.2 M NaCl or 0.2 M choline chloride. The $Na^+$ concentration of a 1.2 mL thrombin sample in choline chloride was varied from 0 to 104 mM by successively removing 15−100 $\mu$L aliquots of the solution and replacing them with an equal volume of the thrombin sample in NaCl. The protein fluorescence was monitored after each $Na^+$ addition using an SLM 8000 spectrofluorometer with an excitation wavelength of 280 nm and an emission wavelength of 340 nm. Data were computer-fit by nonlinear regression to the equation:

$$F_{obs} = (F_o K_D + F_\infty[Na^+])/(K_D + [Na^+])$$

where $F_o$ is the initial fluorescence, $F_\infty$ is the fluorescence at saturating $Na^+$, and $K_D$ is the dissociation constant for $Na^+$ binding. $F_o$, $F_\infty$, and $K_D$ were the fitted parameters.

## RESULTS

The recombinant proteins were expressed, purified, and activated as described under Materials and Methods. The enzymatic activity of thrombin K60fA toward S2238, S2266, and tosyl-GPR-7-amido-4-methylcoumarin synthetic substrates was similar to thrombin, indicating that the mutation did not have an adverse effect on folding or reactivity of the catalytic residues in the K60fA mutant. $K_M$ and $k_{cat}$ values (±2 SE) measured for cleavage of S2238, respectively, were 1.0 ± 0.4 $\mu$M, 77 ± 10 $s^{-1}$ for wild-type thrombin and 1.5 ± 0.7 $\mu$M, 76 ± 20 $s^{-1}$ for K60fA thrombin. These values for cleavage of S2266 were 195 ± 16 $\mu$M, 8.9 ± 0.4 $s^{-1}$ for wild-type thrombin and 202 ± 12 $\mu$M, 15.5 ± 0.2 $s^{-1}$ for K60fA thrombin. The same values for cleavage of the coumarin substrate were 4.7 ± 0.5 $\mu$M, 116 ± 4 $s^{-1}$ for wild-type thrombin and 4.7 ± 0.4 $\mu$M, 98 ± 2 $s^{-1}$ for K60fA thrombin.

To determine whether the Lys60f → Ala mutation broadened the S1′ site specificity of thrombin from just small polar P1′ side chains to include also large hydrophobic side chains, we investigated the reactions of thrombin and K60fA thrombin with a protein inhibitor (antithrombin-Denver) and substrate (protein C in the absence of thrombomodulin) which are poorly recognized by thrombin due to their large apolar P1′ residue, Leu. The natural P1′ Ser → Leu variant, antithrombin-Denver, inhibited thrombin with a 215-fold reduced second-order rate constant [$k = (1.3 \pm 0.1) \times 10^5$ $M^{-1}$ $s^{-1}$] compared to wild-type antithrombin [$k = (2.8 \pm 0.1) \times 10^7$ $M^{-1}$ $s^{-1}$] in the presence of the effector, heparin, in agreement with previous results (Olson et al., 1995). In contrast, antithrombin-Denver inhibited K60fA thrombin under the same conditions with a rate constant [$k = (4.2 \pm 0.1) \times 10^5$ $M^{-1}$ $s^{-1}$] that was only 26-fold slower than wild-type antithrombin [$k = (1.1 \pm 0.1) \times 10^7$ $M^{-1}$ $s^{-1}$]. Comparisons of the individual rate constants indicate that antithrombin-Denver inhibits K60fA thrombin 3.2-fold better than wild-type thrombin. There is, however, a 2.5-fold decrease in the rate constant for wild-type antithrombin inhibition of the K60fA thrombin relative to wild-type thrombin. Taking the impairment in the reactivity of K60fA thrombin into consideration, there is an overall 8.3-fold improvement in the recognition of the P1′ Leu residue of antithrombin-Denver by K60fA thrombin.

Antithrombin inhibits thrombin by a two-step reaction mechanism in which an enzyme−inhibitor encounter complex is initially formed which is then converted to a stable, covalent complex (Olson & Shore, 1982). The P1′ mutation in antithrombin-Denver primarily affects the second step of this two-step reaction (Olson et al., 1995). To determine whether the improved rate of antithrombin-Denver inhibition of K60fA thrombin relative to wild-type thrombin was also due to an effect on the second reaction step, we measured the saturable dependence of pseudo-first-order inhibition rate constants ($k_{obs}$) on inhibitor concentration for antithrombin and antithrombin-Denver reactions with thrombin and K60fA thrombin. These reactions were done in the presence of the

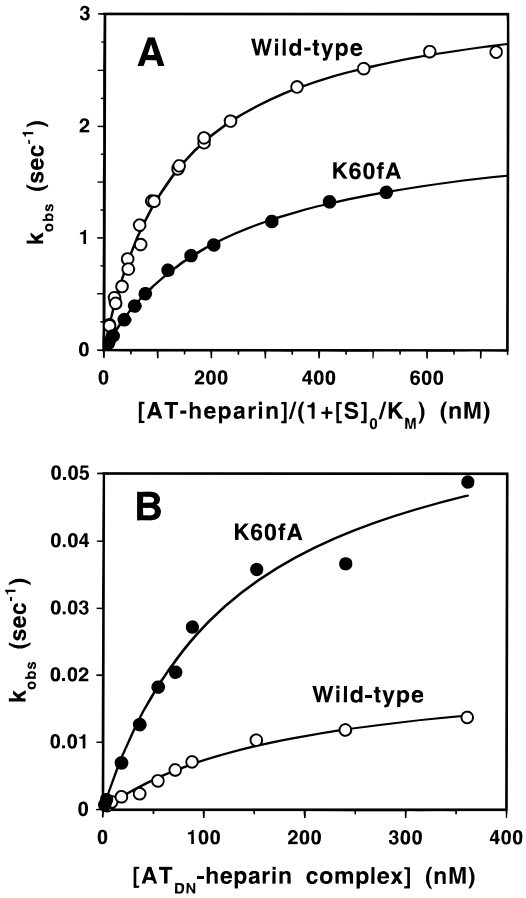


FIGURE 1: Inhibitor concentration dependence of the rates of thrombin and thrombin K60fA inhibition by wild-type antithrombin−heparin complex (panel A) and antithrombin-Denver−heparin complex (panel B). The pseudo-first-order rate constants, $k_{obs}$, for inhibition of wild-type thrombin (○) and thrombin K60fA (●) were measured by continuous or discontinuous assays as described under Materials and Methods. The inhibitor concentration was divided by $1 + [S]_o/K_M$ in the continuous assay to correct for substrate competition. Solid lines are nonlinear regression fits to a rectangular hyperbola.

Scheme 1

$$\text{AT–H} + \text{T} \underset{}{\overset{140 \pm 20\ \text{nM}}{\longleftrightarrow}} \underset{\bullet\,\text{H}\,\bullet}{\text{AT–T}} \xrightarrow{3.2 \pm 0.1\ \text{s}^{-1}} \text{AT–T} + \text{H}$$

$$\text{AT}_{DN}\text{–H} + \text{T} \overset{190 \pm 60\ \text{nM}}{\longleftrightarrow} \underset{\bullet\,\text{H}\,\bullet}{\text{AT}_{DN}\text{–T}} \xrightarrow{0.022 \pm 0.003\ \text{s}^{-1}} \text{AT}_{DN}\text{–T} + \text{H}$$

$$\text{AT–H} + \text{T}_{K60fA} \overset{240 \pm 30\ \text{nM}}{\longleftrightarrow} \underset{\bullet\,\text{H}\,\bullet}{\text{AT–T}_{K60fA}} \xrightarrow{2.1 \pm 0.1\ \text{s}^{-1}} \text{AT–T}_{K60fA} + \text{H}$$

$$\text{AT}_{DN}\text{–H} + \text{T}_{K60fA} \overset{140 \pm 40\ \text{nM}}{\longleftrightarrow} \underset{\bullet\,\text{H}\,\bullet}{\text{AT}_{DN}\text{–T}_{K60fA}} \xrightarrow{0.065 \pm 0.009\ \text{s}^{-1}} \text{AT}_{DN}\text{–T}_{K60fA} + \text{H}$$

activator heparin so that saturation of the initial enzyme−inhibitor complex could be observed in the range of inhibitor concentrations accessible to measurement (Figure 1). Nonlinear least-squares analysis of the hyperbolic saturation curves obtained for all reactions yielded the values for the ternary complex dissociation constant and the rate constant for stable complex formation (±2 SE) shown in Scheme 1. The decreased rate of antithrombin inhibition of K60fA thrombin relative to thrombin resulted partly from an increased ternary complex dissociation constant (1.7-fold) and partly from a decreased rate constant for formation of stable complex (1.5-fold). In contrast, the enhanced rate of antithrombin-Denver inhibition of the mutant thrombin relative to wild-type enzyme was mostly localized in the second step; i.e., the rate constant for formation of stable complex was enhanced ~3-fold with no distinguishable effect on the ternary complex formation step within experimental error. Comparison of the rate constants for the second reaction step further showed that this rate constant is reduced ~145-fold by the Denver mutation in the reaction of antithrombin with wild-type thrombin, whereas this rate constant is reduced only 32-fold by the Denver mutation in the reaction of the inhibitor with K60fA thrombin. Overall, the results indicate that the partial restoration of the functional defect of antithrombin-Denver occurs largely in the second step of the reaction with only a minor effect on the first step.

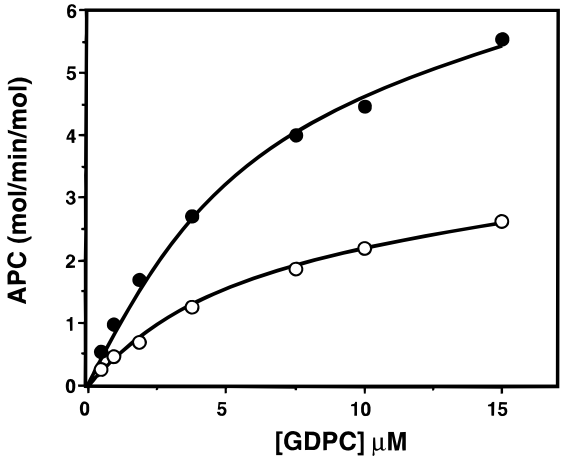


FIGURE 2: Kinetic analysis of Gla-domainless protein C activation by thrombin and thrombin K60fA in the presence of TM. The initial rates of activation were determined as a function of GDPC concentration with 1 nM thrombin (○) or thrombin K60fA (●) in complex with 100 nM TM4-6 in TBS buffer containing 1 mg/mL BSA and 2.5 mM $Ca^{2+}$ at room temperature as described under Materials and Methods. The kinetic constants, $K_M$ and $k_{cat}$, were determined from nonlinear regression fits to the Michaelis−Menten equation (solid lines).

Comparisons of the initial rate of Gla-domainless protein C activation by wild-type and mutant thrombins indicated that thrombin K60fA activates protein C ~3-fold faster than thrombin in the presence of EDTA and ~4-fold faster in the presence of 5 mM $Ca^{2+}$ (data not shown). Thrombin K60fA activated GDPC ~2-fold better than thrombin in the presence of TM4-6 and $Ca^{2+}$ (data not shown). Kinetic analysis (Figure 2) showed that thrombin in the presence of TM4-6 activated GDPC with a $K_M$ of 7.8 ± 1.1 $\mu$M and a $k_{cat}$ of 0.07 ± 0.01 $s^{-1}$. The same values with K60fA activation of GDPC were $K_M = 6.7 \pm 0.7$ $\mu$M and $k_{cat} = 0.14 \pm 0.02$ $s^{-1}$. Consistent with the results obtained above, the improvement in the rate of protein C activation by K60fA resulted from an increased rate constant for the second reaction step. A detailed kinetic analysis in the absence of TM was not possible, since thrombin in the absence of TM exhibits a high $K_M$ for GDPC both in the presence and in the absence of $Ca^{2+}$.

In contrast to the improvements observed in the inhibition of K60fA thrombin by antithrombin-Denver and in the activation of protein C by K60fA thrombin relative to these same reactions with wild-type thrombin, thrombin K60fA clotted fibrinogen slower than wild-type thrombin. Under conditions well below the $K_M$ for fibrinogen where fibrinopeptide cleavage by thrombin is rate-limiting with respect to the subsequent aggregation of fibrin (De Cristofaro & Di

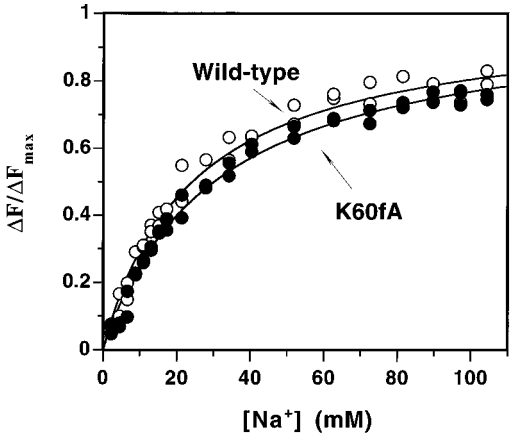


FIGURE 3: Binding of $Na^+$ to wild-type and K60fA thrombins. 100 nM wild-type (○) and K60fA (●) thrombins in 5 mM Tris-HCl, 0.18 M choline chloride, and 0.1% PEG 8000, pH 8.0, 25 °C, were titrated with an equivalent concentration of enzyme in buffer containing 0.18 M NaCl in place of choline chloride. Binding of $Na^+$ was monitored from changes in the intrinsic protein fluorescence. Details are provided under Materials and Methods. Solid lines are fits to the binding equation given in the text which indicated maximal relative fluorescence enhancements of 17 ± 1% for both enzymes.

Cera, 1991), (3.5 ± 0.1)-fold more K60fA thrombin was required to yield the same fibrinogen clotting time as wild-type thrombin in an assay using pure fibrinogen. Similarly, 3−3.5-fold more K60fA thrombin than wild-type thrombin was required to produce an equivalent clotting time in a standard clotting assay using human plasma or pure fibrinogen at concentrations exceeding the fibrinogen $K_M$. The parallel plots of log clotting time vs log thrombin dilution obtained for wild-type and mutant thrombins in each assay indicated that these results were independent of thrombin dilution.

Thrombin contains a $Na^+$ binding site whose occupancy allosterically modulates the activity of thrombin (Wells & Di Cera, 1992). Thrombin in the $Na^+$-bound form (fast form) preferentially cleaves fibrinogen whereas in the $Na^+$-free form (slow form) thrombin preferentially activates protein C (Dang et al., 1995). The slow form of thrombin also reacts with antithrombin at a reduced rate relative to the fast form (Di Cera et al., 1995). Since the increased reactivity of K60fA thrombin in protein C activation but decreased reactivity in fibrinogen clotting and in antithrombin inhibition in the presence of heparin resembled the properties of the slow form of thrombin (Di Cera et al., 1995), we determined whether the K60fA mutation stabilized the slow form by decreasing the affinity of the enzyme for $Na^+$. Equilibrium binding titrations of $Na^+$-free forms of wild-type and K60fA thrombins with $Na^+$ monitored by the ∼20% enhancement in intrinsic protein fluorescence which accompanies $Na^+$ binding to the enzyme (Wells & Di Cera, 1992) indicated that the K60fA mutation minimally affected $Na^+$ binding (Figure 3). Indistinguishable $K_D$s of 24 ± 6 and 30 ± 5 mM for $Na^+$ binding to wild-type and K60fA thrombins were thus measured from these titrations. Similar values were previously reported for the wild-type enzyme (Wells & Di Cera, 1992). These results indicate that the K60fA mutation does not alter thrombin specificity indirectly by stabilizing the slow form, but rather directly modifies the specificity of the fast form of the enzyme.

The effects of the K60fA mutation on the reactivity of the slow form of thrombin were examined by comparing the rates of thrombin inhibition by antithrombin-Denver and thrombin activation of protein C in 20 mM Tris-HCl buffer containing either 200 mM NaCl or 5 mM NaCl plus 195 mM choline chloride (Dang et al., 1995). Similar ∼3-fold enhancements of the pseudo-first order rate constant for 100 nM antithrombin-Denver inhibition of 4 nM K60fA thrombin vs wild-type thrombin in the presence of 150 nM heparin were observed in both the low- and high-$Na^+$ buffers. Protein C activation was examined at three different concentrations of GDPC (2.5, 5, and 10 $\mu$M) and 1 nM thrombin in complex with 50 nM TM4-6. Although the rate of protein C activation was improved in the low-$Na^+$ buffer relative to the high-$Na^+$ buffer as reported (Dang et al., 1995), the rate of protein C activation by K60fA thrombin was ∼2-fold higher than wild-type thrombin in both low- and high-$Na^+$ buffers. These results indicate that the K60fA mutation improves thrombin recognition of P1′ Leu residues in both slow and fast forms of thrombin.

## DISCUSSION

The S1′ substrate binding site of thrombin makes a significant contribution to the specificity of thrombin for macromolecular substrates and inhibitors (Stubbs & Bode, 1993). Studies of the reactivity of thrombin with natural and engineered P1′ variants of the serpin inhibitor, antithrombin, have demonstrated that the thrombin S1′ site has a marked preference for small polar residues (Stephens et al., 1988; Theunissen et al., 1993). The basis for this S1′ site specificity has been suggested from the X-ray structure of thrombin to result from the occlusion of this site by Lys60f in the B insertion loop (Bode et al., 1989; Theunissen et al., 1993). In support of this hypothesis, the present study demonstrates that substitution of Lys60f with Ala partially corrects the defective inhibition of thrombin by antithrombin-Denver, a natural P1′ Ser → Leu variant, and enhances the reactivity of thrombin toward a natural substrate, protein C, which possesses a P1′ Leu residue. In contrast, the reactivity of the Lys60f → Ala thrombin variant toward fibrinogen or wild-type antithrombin whose small P1′ residue (Gly and Ser, respectively) would be expected to be well accommodated in the S1′ site is modestly reduced. Such findings indicate that the Lys60f residue is at least partly responsible for the inability of the S1′ site to accommodate Leu and presumably also other bulky hydrophobic P1′ residues.

Previous studies have shown that the defective inhibition of thrombin by antithrombin-Denver results from an effect on the second step of a two-step inhibitor protease reaction (Olson et al., 1995). The rate constant for conversion of an initial loose antithrombin−thrombin encounter complex to a stable, covalent complex was thus found to be reduced in the antithrombin-Denver reaction by ∼145-fold. Such findings suggested that, similar to a true substrate (Fersht et al., 1973), the antithrombin P1′ residue interaction contributes to transition-state stabilization of the attack of the catalytic serine residue of thrombin on the inhibitor scissile bond following the formation of a Michaelis-type enzyme−inhibitor complex. In contrast to a true substrate, however, the tetrahedral or acyl-intermediate formed as a result of the catalytic serine attack induces a conformational change in the serpin inhibitor which leads to the trapping of the enzyme in a stable covalent inhibitor−enzyme complex (Olson et

al., 1995). Consistent with these prior findings, the present study has found that the improvement in the reactivity of antithrombin-Denver with thrombin produced by the K60fA mutation is also due to an effect on the second reaction step. Such results support the conclusion that the Lys60f → Ala mutation promotes the P1′−S1′ interaction in the second reaction step by increasing the size of the S1′ specificity site so that it can accommodate larger hydrophobic residues. Our results are thus in agreement with the proposal that the reduction in size of the S1′ site by Lys60f is at least partly responsible for the poor ability of bulky hydrophobic P1′ residues such as Leu to bind in the S1′ site and stabilize the tetrahedral transition state (Theunissen et el., 1993; Olson et al., 1995).

The observation that the reactivity of antithrombin-Denver with K60fA thrombin is still ∼26-fold lower than antithrombin indicates that occlusion of the S1′ site by Lys60f cannot be the only reason for the reduced reactivity of the P1′ Leu variant of antithrombin with thrombin. Thus, while the size of the S1′ binding site may be increased by the K60fA mutation to allow binding of larger amino acid side chains, the site may still retain a hydrophilic character in the K60fA variant due to other residues which line this binding pocket and thereby make the binding of hydrophobic residues still unfavorable (Stubbs & Bode, 1993). Moreover, the Leu residue may not optimally fit in the larger S1′ site of the mutant thrombin. The P1′ Leu residue may therefore not match the size and hydrophilicity requirements of the modified S1′ site of K60fA thrombin (Stephens et al., 1988).

Our finding that the K60fA mutation in thrombin reduces the reactivity of the enzyme with wild-type antithrombin 2.5-fold and the clotting activity with the substrate, fibrinogen, by ∼3-fold indicates modest negative effects of the mutation on inhibitor and substrate binding. Changes in the size and hydrophilicity of the S1′ site of the mutant thrombin may contribute to these negative effects. Interestingly, mutation of the Lys60f residue of thrombin to Glu (K60fE) in a previous study reduced the reactivity of thrombin with antithrombin in the absence but not the presence of heparin, suggesting a dependence of the mutation′s effect on the polysaccharide activator (Sheehan et al., 1993).

The K60fA mutation in thrombin reduced fibrinogen clotting activity by 3−3.5-fold at fibrinogen concentrations both well below $K_M$ and well above $K_M$. An effect of the mutation on the $k_{cat}$ of fibrinogen cleavage by thrombin could explain these observations, since $k_{cat}$ determines the rate of fibrinogen cleavage both at low and at high concentrations of substrate and could thereby limit the fibrinogen clotting time in either range of concentration. Because of the importance of a main chain P2′ amide hydrogen bond interaction with the S2′ site in productive enzyme−substrate interactions and the inability of the P2′ Pro of fibrinogen to make such a hydrogen bond, the main chain carbonyl of the P2′ Pro has been proposed to hydrogen bond to the Lys60f side chain (Stubbs & Bode, 1993). The inability of the Ala60f residue in the mutant thrombin to form a similar hydrogen bond could explain the negative effect of the mutation on the $k_{cat}$ step for fibrinogen cleavage and would be consistent with steps observed to be affected by the K60fA mutation in reactions with antithrombin and protein C. This possibility is supported by the similar negative effects of a K60fE mutation on fibrinogen clotting (6-fold reduced clotting activity) reported in a previous study (Wu et al., 1991).

Protein C resembles antithrombin-Denver in that the P1′ residue for activation of this substrate is also Leu which is thus not expected to fit the S1′ pocket of thrombin optimally. The rate of protein C activation by K60fA thrombin in the absence and presence of TM was found to be 4- and 2-fold faster, respectively, than wild-type thrombin. The improvement in activation in the presence of TM was localized to the second step of the reaction, i.e., in the $k_{cat}$ for protein C activation, similar to the step affected in antithrombin-Denver reactions with wild-type and variant thrombins. Because of a high $K_M$ value for protein C activation by both wild-type and K60fA thrombins in the absence of TM, we were unable to determine which step of the activation was improved in the absence of cofactor. However, the $k_{cat}$ is likely to also be the step affected in this reaction if an improved interaction of the P1′ Leu with the larger S1′ site of the mutant thrombin is responsible for the enhanced activation, since the P1′−S1′ interaction is expected to stabilize the transition state for substrate bond cleavage (Fersht et al., 1973).

In the case of protein C, it appears that conservation of nonoptimal residues near the thrombin cleavage site has important physiological implications for the regulation of protein C activation. Both the P3 and P3′ residues of protein C are Asp residues which are also not optimal for recognition by thrombin (Rezaie & Esmon, 1992). When thrombin binds to thrombomodulin on the surface of endothelial cells, it can no longer clot fibrinogen or activate platelets, but is transformed into an efficient activator of protein C. This dramatic alteration in thrombin specificity results from a conformational change in the active-site pocket which alleviates the inhibitory effect of interactions with nonoptimal residues flanking the scissile bond (Esmon, 1993). These residues therefore play an important role in preventing unregulated protein C activation by thrombin alone and may be linked to evolution of an intricate molecular switch system which enables thrombin to function in both procoagulant and anticoagulant systems. In contrast to the significant role that residue Glu192 plays in this molecular switch system (Esmon, 1993), the role of Lys60f appears to be modest since the rate of protein C activation by thrombin K60fA was only 2−4-fold faster than thrombin. However, the inability of the K60fA mutation to fully restore wild-type reactivity with antithrombin-Denver suggests that the extent to which the P1′ Leu contributes to this molecular switch may be underestimated. It is also noteworthy that a greater enhancement in protein C activation by K60fA thrombin relative to thrombin was observed in the absence of TM than in the presence of the cofactor, consistent with TM alleviating the unfavorable S1′ interaction with the P1′ Leu so that the K60fA mutation produces a smaller effect. A similar 2−3-fold improvement in protein C activation was observed with K60fE thrombin either in the absence of both TM and $Ca^{2+}$ or in the presence of the two effectors (Wu et al., 1991). However, the same study found no improvement in activation in the absence of TM when $Ca^{2+}$ was present, in contrast to the 4-fold improved activation observed in the present study under these conditions. However, the large experimental errors reported for the slow activation rates measured under the latter conditions may have obscured a small activating effect.

The improved specificity of K60fA thrombin for a substrate (protein C) and an inhibitor (antithrombin-Denver) with nonoptimal P1′ Leu residues was not an indirect result of altering the allosteric transition of thrombin regulated by the binding of $Na^+$ (Wells & Di Cera, 1991). Relative to the $Na^+$-bound or fast form of thrombin which was the predominant species under the conditions used in our studies, the $Na^+$-free or slow form of the enzyme has enhanced reactivity with protein C but reduced reactivity with fibrinogen and antithrombin (Di Cera et al., 1995), similar to the behavior of the K60fA thrombin. However, $Na^+$ binding to the mutant thrombin was normal, indicating that the mutation does not perturb the $Na^+$ binding site. Comparisons of the effects of the K60fA mutation on thrombin reactivity with antithrombin-Denver and protein C further indicated that the K60fA mutation improves thrombin recognition of P1′ Leu residues in both fast and slow forms of the enzyme. Although different conformations of the Lys60f side chain have been proposed to exist in the slow and fast forms of thrombin based on the differential recognition of hirudin by the two thrombin forms (Ayala et al., 1995), our results suggest that both Lys60f conformations sterically interfere with binding of P1′ Leu residues and that this interference can be overcome by the K60fA mutation in both forms of thrombin.

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## REFERENCES

Ayala, Y. M., Vindigni, A., Nayal, M., Spolar, R. S., Record, M. T., Jr., & Di Cera, E. (1995) *J. Mol. Biol. 253*, 787−798.
Björk, I., Ylinenjärvi, K., Olson, S. T., Hermentin, P., Conradt, H. S., & Zettlmeissl, G. (1992) *Biochem. J. 286*, 793−800.
Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S. R., & Hofsteenge, J. (1989) *EMBO J. 8*, 3467−3475.
Bode, W., Turk, D., & Karshikov, A. (1992) *Protein Sci. 1*, 426−471.
Cooper, S. T., & Church, F. C. (1995) *Biochim. Biophys. Acta 1246*, 29−33.
Dang, Q. D., Vindigni, A., & Di Cera, E. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 5977−5981.
De Cristofaro, R., & Di Cera, E. (1991) *J. Protein Chem. 10*, 455−468.
Di Cera, E., Guinto, E. R., Vindigni, A., Dang, Q. D., Ayala, Y. M., Wuyi, M., & Tulinsky, A. (1995) J. *Biol. Chem. 270*, 22089−22092.
Esmon, C. T. (1989) *J. Biol. Chem. 264*, 4743−4746.
Esmon, C. T. (1993) *Thromb. Haemostasis 70*, 1−5.
Fersht, A. R., Blow, D. M., & Fastrez, J. (1973) *Biochemistry 12*, 2035−2041.
Gan, Z.-R., Li, Y., Chen, Z., Lewis, D. D., & Shafer, J. A. (1994) *J. Biol. Chem. 269*, 1301−1305.
Guinto, E. R., Ye, J., Le Bonniec, B. F., & Esmon, C. T. (1994) *J. Biol. Chem. 269*, 18395−18400.
Heeb, M. J., Bischoff, R., Courtney, M., & Griffin, J. H. (1990) *J. Biol. Chem. 265*, 2365−2369.
Higuchi, R., Krummel, B., & Saiki, R. (1988) *Nucleic Acids Res. 16*, 7351−7367.
Hopkins, P. C. R., Crowther, D. C., Carrell, R. W., & Stone, S. R. (1995) *J. Biol. Chem. 270*, 11866−11871.
Huber, R., & Bode, W. (1978) *Acc. Chem. Res. 11*, 114−122.
Jakubowski, H. V., & Owen, W. G. (1989) *J. Biol. Chem. 264*, 11117−11121.
Le Bonniec, B. F., Guinto, E. R., & Esmon, C. T. (1992) *J. Biol. Chem. 267*, 19341−19348.
Le Bonniec, B. F., Guinto, E. R., MacGillivray, R. T. A., Stone, S. R., & Esmon, C. T. (1993) *J. Biol. Chem. 268*, 19055−19061.
Lottenberg, R., Hall, J. A., Blinder, M., Binder, E. P., & Jackson, C. M. (1983) *Biochim. Biophys. Acta 742*, 539−557.
Mann, K. G., Williams, E. B., Krishnaswamy, S., Church, W., Giles, A., & Tracy, R. P. (1990) *Blood 76*, 755−766.
Olson, S. T., & Shore, J. D. (1982) *J. Biol. Chem. 257*, 14891−14895.
Olson, S. T., Björk, I., & Shore, J. D. (1993) *Methods Enzymol. 222*, 525−559.
Olson, S. T., Stephens, A. W., Hirs, C. H. W., Bock, P. E., & Björk, I. (1995) *J. Biol. Chem. 270*, 9717−9724.
Rezaie, A. R. (1996) *Biochemistry 35*, 1918−1924.
Rezaie, A. R., & Esmon, C. T. (1992) *J. Biol. Chem. 267*, 26104−26109.
Rezaie, A. R., & Esmon, C. T. (1994) *Blood 83*, 2526−2531.
Rydel, T. J., Ravichandran, K. G., Tulinsky, A., Bode, W., Huber, R., Roitsch, C., & Fenton, J. W., II (1990) *Science 249*, 277−280.
Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157−162.
Segal, I. H. (1975) *Enzyme Kinetics*, pp 59−61, John Wiley and Sons, New York.
Sheehan, J. P., Wu, Q., Tollefsen, D. M., & Sadler, J. E. (1993)) *J. Biol. Chem. 268*, 3639−3645.
Stephens, A. W., Siddiqui, A., & Hirs, C. H. W. (1988) *J. Biol. Chem. 263*, 15849−15852.
Stubbs, M. T., & Bode, W. (1993) *Thromb. Res. 69*, 1−58.
Theunissen, H. J. M., Dijikema, R., Grootenhuis, P. D. J., Swinkels, J. C., DePoorter, T. L., Carati, P., & Visser, A. (1993) *J. Biol. Chem. 268*, 9035−9040.
Wells, C. M., & Di Cera, E. (1992) *Biochemistry 31*, 11721−11730.
Wu, Q., Sheehan, J. P., Tsiang, M., Lentz, S. R., Birktoft, J. J., & Sadler, J. E. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6775−6779.
Ye, J., Rezaie, A. R., & Esmon, C. T. (1994) *J. Biol. Chem. 269*, 17965−17970.

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