Protein Engineering Thrombin for Optimal Specificity and Potency of Anticoagulant Activity *in Vivo*

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Received July 3, 1996; Revised Manuscript Received September 16, 1996⊗

ABSTRACT: Previous alanine scanning mutagenesis of thrombin revealed that substitution of residues W50, K52, E229, and R233 (W60d, K60f, E217, and R221 in chymotrypsinogen numbering) with alanine altered the substrate specificity of thrombin to favor the anticoagulant substrate protein C. Saturation mutagenesis, in which residues W50, K52, E229, and R233 were each substituted with all 19 naturally occurring amino acids, resulted in the identification of a single mutation, E229K, that shifted the substrate specificity of thrombin by 130-fold to favor the activation of the anticoagulant substrate protein C over the procoagulant substrate fibrinogen. E229K thrombin was also less effective in activating platelets (18-fold), was resistant to inhibition by antithrombin III (33-fold and 22-fold in the presence and absence of heparin), and displayed a prolonged half-life in plasma *in vitro* (26-fold). Thus E229K thrombin displayed an optimal phenotype to function as a potent and specific activator of endogenous protein C and as an anticoagulant *in vivo*. Upon infusion in Cynomolgus monkeys E229K thrombin caused an anticoagulant effect through the activation of endogenous protein C without coincidentally stimulating fibrinogen clotting and platelet activation as observed with wild-type thrombin. In addition, E229K thrombin displayed enhanced potency *in vivo* relative to the prototype protein C activator E229A thrombin. This enhanced potency may be attributable to decreased clearance by antithrombin III, the principal physiological inhibitor of thrombin.

Activation of the multienzyme blood coagulation cascade results in the conversion of the inactive zymogen prothrombin into active thrombin (Rosenberg, 1987; Jackson & Nemerson, 1980). Thrombin is a multifunctional serine protease that recognizes and proteolytically activates multiple substrates (Mann & Lundblad, 1987; Esmon 1987) that have both procoagulant and anticoagulant functions. Through precise regulation of its substrate specificity thrombin may function as a promoter of blood coagulation, mediating both fibrin clot formation and platelet aggregation, or alternatively thrombin may act as a regulator of coagulation by activating a natural anticoagulant mechanism, the protein C (PC) pathway.

At sites of vascular injury or inflammation, thrombin recognizes two substrates with key procoagulant functions. The hydrolysis of fibrinogen results in the formation of an insoluble fibrin clot, and the cleavage of the platelet thrombin receptor results in the generation of a tethered ligand that promotes platelet activation and aggregation (Vu et al., 1991). Additional procoagulant substrates include factor XIII (Lorand & Radek, 1992), which stabilizes the clot by cross-linking fibrin monomers, and factors V, VIII (Mann et al., 1988), and XI (Gailani & Broze, 1991) that perpetuate the clotting stimulus and the generation of new thrombin through feedback activation of the coagulation cascade. Thrombin that escapes the site of an injury becomes bound to the cofactor thrombomodulin (TM), a transmembrane protein expressed on endothelial cells that line the vasculature. Upon binding to TM the activity of thrombin toward the procoagulant substrates is blocked, and a proposed Ca^{2+}-dependent conformational change enhances the recognition and activation of the anticoagulant substrate PC. Activated protein C (aPC) cleaves and inactivates activated coagulation factors Va and VIIIa to repress the coagulation cascade and may also enhance fibrinolysis by inactivating PAI-1, the principal physiological inhibitor of tissue plasminogen activator (Comp & Esmon, 1981; van Hinsbergh, 1985), or indirectly by inhibiting the activation of TAFI (thrombin activatable inhibitor of thrombolysis) (Bajzar et al., 1995). Thus the aPC pathway may serve as a mechanism to attenuate the extent of blood clotting and prevent propagation of the clot beyond the site of an injury (Esmon, 1987).

Several approaches have been used to exploit the PC pathway as a means to achieve anticoagulation *in vivo* including infusion of soluble thrombomodulin (Gomi et al., 1990), infusion of a PC-activating protease isolated from the venom of snakes belonging to the genus *Akistrodon* (Strukova et al., 1989; Kogan et al., 1993), direct infusion of exogenously activated PC (Comp & Esmon, 1981; Gruber et al., 1989, 1990; Okajima et al., 1990; Taylor et al., 1987), or infusion of a variant of PC that is more susceptible to activation by thrombin (Richardson et al., 1992; Kurz et al., 1994).

An alternative approach has been to take advantage of enzyme turnover and activate endogenous PC by infusion of thrombin itself. Infusion of thrombin in dogs resulted in systemic anticoagulation (Comp et al., 1982), and infusion of thrombin in baboons was shown to be effective in reducing thrombus formation in arteriovenous shunts (Hanson et al., 1993). In both cases, coinfusion of antibodies that blocked PC also blocked the anticoagulant effect of the thrombin

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⊗ Abstract published in *Advance ACS Abstracts,* December 1, 1996.

S0006-2960(96)01610-8 CCC: $12.00

infusion, strongly implicating the aPC anticoagulant pathway. However, the effects of the procoagulant activities of thrombin were also evident particularly at higher doses. Elevation of markers of fibrin formation and platelet activation and consumption of fibrinogen and clotting factors were indicative of intravascular coagulation.

One approach to minimizing the procoagulant functions of thrombin while retaining the anticoagulant effect has been to infuse a reversibly acylated form of thrombin [(guanidinobenzoyl)thrombin] that was maintained in inactive form prior to thrombomodulin binding (McBane et al., 1995). However, preliminary studies demonstrating that the PC activation and fibrinogen clotting activities of thrombin could be dissected by point mutations (Wu et al., 1991) suggested to us that a genetically engineered variant of human thrombin with impaired specificity for the procoagulant substrates that retained anticoagulant activity might be a more attractive anticoagulant agent.

A library of 62 thrombin mutants, in which the charged and polar amino residues that were highly exposed on the surface of the molecule were substituted with the small neutral amino acid alanine, was screened for mutants that were defective in fibrinogen clotting yet retained their ability to activate PC in the presence of TM. Four mutants (W50A, K52A, E229A, and R233A) displayed significant shifts in their substrate specificity that favored the anticoagulant substrate, PC (Tsiang et al., 1995). The E229A mutant, which displayed a 22-fold switch in substrate specificity, was selected as a prototype and was demonstrated to function as a selective endogenous protein C activator (PCA) in Cynomolgus monkeys, causing reversible anticoagulation without activating platelets or fibrin clot formation (Gibbs et al., 1995).

In this study, we attempted to optimize the specificity of thrombin toward PC by individually substituting thrombin residues W50, K52, E229, and R233 with all 19 naturally occurring amino acids. Screening of this collection of all 76 possible substitutions identified a mutant, E229K, with enhanced specificity for PC and enhanced anticoagulant potency *in vivo*.

EXPERIMENTAL PROCEDURES

Materials. Reacti-BIND maleic anhydride activated polystyrene 96-well plates were from Pierce (catalog no. 15110). PPACK (D-Phe-Pro-Arg chloromethyl ketone) was from Sigma. Purified human α-thrombin and protein C (free of detectable prothrombin) were from Haematologic Technologies. Purified human antithrombin III was from Enzyme Research Laboratories. The anti-human prothrombin polyclonal antibody was from DAKO (catalog no. 325), and the goat anti-rabbit antibody conjugated with horseradish peroxidase was from Vector Laboratories. *Echis carinatus* snake venom was from Sigma. DEAE-Sepharose Fast Flow was from Pharmacia. Amberlite CG50 cation-exchange resin was from ICN Biomedicals.

Construction, Transient Expression, and Screening of Thrombin Mutants. The detailed methods describing the expression, processing, and assay of transiently expressed thrombin mutants have been described previously (Tsiang et al., 1995).

Thrombin mutants were constructed by oligonucleotide-directed mutagenesis as described previously (Gibbs & Zoller, 1991) using four synthetic oligonucleotide primers where the codons encoding thrombin residues W50, K52, E229, and R233 were replaced with randomized codons NNS (where N = A or C or G or T and S = C or G). On average, clones encoding 13/19 of all possible amino acid substitutions at each position were identified following the sequencing of 48 clones. The remaining mutations were constructed using specific mutagenesis primers. The collection of 76 thrombin mutants was transiently expressed in COS-7 cells. Conditioned medium was concentrated by ultrafiltration and treated with *E. carinatis* venom to process prothrombin to thrombin. Processing was determined to be complete following analysis by Western blotting of reducing SDS−PAGE gels. Thrombin concentration in concentrated conditioned medium was determined by ELISA (see below). Specific amidolytic activity [S-2238 (H-D-Phe-Pip-Arg-pNA) hydrolysis], fibrinogen clotting activity, and PC activation in the presence of TM were determined as described previously (Tsiang et al., 1995). Specific fibrinogen clotting activity (FC) (expressed as a percent of wild-type thrombin activity) was determined from the time to clot formation following the addition of purified fibrinogen (to 0.4 mg/mL) to a predetermined amount of each thrombin mutant. Clotting activity was related to the clotting activity of wild-type thrombin using a standard curve of clotting time relative to thrombin concentration. Specific activity of protein C activation (PA) (expressed as a percent of wild-type thrombin activity) was determined by incubating each thrombin mutant with 5 nM TM and 887 nM PC. The concentration of aPC generated was determined from the hydrolysis of the chromogenic substrate S2366 following termination of the reaction by the addition of heparin/antithrombin III. Specific activity of PC activation was based on the concentration of thrombin/TM complexes in the reaction. The relative specificity for protein C activation over fibrinogen clotting was determined from the PA/FC ratio.

Thrombin ELISA. The expression level of each thrombin mutant was estimated using an immunoassay employing PPACK to capture and immobilize thrombin on a microtiter plate for subsequent detection using an anti-human prothrombin/thrombin polyclonal antibody. Purified human α-thrombin was used to construct a standard curve for each assay. Microtiter plates were prepared freshly for each assay as follows: PPACK (15 μM) in PBS was coated onto the bottom of a maleic anhydride activated 96-well plate and incubated overnight at room temperature. The PPACK was removed, and the excess sites on the plate were blocked for 30 min with 3% BSA in PBS and 0.05% Tween-20 (Tw). The blocking step was repeated, and the plate was then washed with wash buffer (PBS, 0.05 % Tw). Triplicate aliquots of purified thrombin standards (0.5−12 ng/mL) and duplicate aliquots of thrombin mutants were diluted in dilution buffer (0.1% BSA in PBS 0.05% Tw) and then added to the plate and incubated for 2 h at room temperature. Thrombin was detected by incubating the plate for 1 h at room temperature with the rabbit anti-human prothrombin/thrombin polyclonal antibody (1:1000) diluted in dilution buffer followed by 1 h incubation at room temperature with a goat anti-rabbit antibody conjugated with horseradish peroxidase (1:2000) diluted in PBS and 0.1% Tw. The plate was washed between each incubation step and developed using the peroxidase substrate ABTS (Vector Laboratories). The absorbance at 405 nm was determined using a Molecular Devices plate reader. The concentration of each thrombin mutant was determined from the standard curve using data

from at least two dilutions each measured in duplicate. The assay was sensitive to 0.5 ng/mL thrombin, and the linear range extended beyond 12 ng/mL.

Expression of Selected Thrombin Mutants Using an Expression Vector Based on the Sindbis Virus. An *Xba*I fragment encoding the entire prothrombin coding region was isolated from BS(KS)-hFII (obtained from Ross MacGillivray, University of British Columbia) (Friezner Degen et al., 1983) and was cloned into the *Xba*I site downstream of the subgenomic RNA promoter in the Sindbus virus-based expression vector SINrep5 (Bredenbeek et al., 1993) to generate SINrep5-hPT. A 1301 bp *Kas*I−*Apa*I fragment that spans the region encoding the thrombin B-chain was isolated from pRc/CMV-hPT clones expressing selected mutants (E229A, E229K, E229S, E229W, E229Y, R233F, W50E, W50I) and used to replace the corresponding *Kas*I−*Apa*I fragment in SINrep5-hPT, allowing expression of thrombin mutants from the Sindbis subgenomic promoter.

Cell Culture and Cell Lines. COS-7 and BHK-21 cells were grown and passaged in media I (DMEM containing 4.5 mg/mL glucose, 3.7 mg/mL $NaHCO_3$, 10% CS, 2 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin). During the prothrombin secretion stage, BHK-21 cells were incubated with media II (serum-free media I supplemented with 10 μg/mL vitamin K, 5 μg/mL insulin, 5 μg/mL transferrin, and 5 μg/mL fetuin). The replicon packaging helper cell line BHK-BBneo was a generous gift from Dr. Sondra Schlesinger (Washington University, St. Louis, MO). This cell line was derived by stable transfection of BHK-21 cells (Bredenbeek et al., 1993) with a construct comprised of the Sindbis helper RNA (DH-BB) encoding the Sindbis structural proteins (Liljeström & Garoff, 1991; Liljeström et al., 1991) followed by a Neo^R gene. In the absence of Sindbis replication machinery (supplied in *trans* through transfection of replicon RNAs), these cells do not express Sindbis-specific polypeptides. BHK-BBneo cells were passaged in media III [α-MEM containing 10% FCS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 800 μg/mL G418 (Gibco)].

Generation of Sindbis Replicons and Expression of Prothrombin Mutants. SINrep5-hPT mutants were linearized with *Not*I. The linearized plasmids (1−3 μg/50 μL of reaction) were used as template for the synthesis of replicon RNA by SP6 RNA polymerase in the presence of 1 mM 5′ cap analog $m^7G(5')ppp(5')G$ (New England Biolabs) as previously described (Rice et al., 1987). BHK-BBneo cells were transfected with 2 pmol of replicon RNA by electroporation, diluted in 13.5 mL of media III, and plated in two T25 flasks (Liljeström et al., 1991). The media containing packaged replicons were harvested 48 h postelectroporation when the cytopathic effect was obvious. At this time, titers of packaged replicons were 3×10^8−2×10^9 infectious units/mL (Schlesinger, personal communication). We assumed that all our P0 harvests had a titer of $\sim 1 \times 10^8$ infectious units/mL. To amplify the replicon stock, new monolayers of BHK-BBneo were infected with the P0 harvest at an moi of ∼1.0. The amplified replicon stock harvested 48 h postinfection was used to infect BHK-21 cells in roller bottles at an moi of ∼6.0. Seventy two hours postinfection, conditioned medium containing secreted prothrombin was harvested. The expression level was ∼4.6 μg of prothrombin/10^6 cells or ∼417 μg of prothrombin/roller bottle.

Purification of Thrombin Mutants. The purification procedures for prothrombin and thrombin were modified from previously described protocols (Wu et al., 1991; Fenton et al. 1977). For each mutant, the conditioned medium from four roller bottles was clarified by centrifugation at 2500 rpm and vacuum filtration through Whatman No. 3 and Whatman No. 1 filter paper. Sindbis replicon particles in the media were inactivated by adding Triton X-100 to a final concentration of 0.2%. Benzamidine hydrochloride (5 mM) and barium sulfate (40 mg/mL) were added, and the medium was incubated on ice for 30 min. The medium was then centrifuged at 3000 rpm for 15 min, and the pellet was washed three times with 5 mM sodium acetate, pH 7.0. The prothrombin was eluted with 200 mM sodium citrate, pH 7.0, and 5 mM benzamidine hydrochloride and dialyzed against 0.1 M potassium phosphate, pH 7.5, containing 5 mM benzamidine hydrochloride with the final dialysis step performed in the absence of benzamidine hydrochloride. The dialysate was then loaded onto a DEAE-Sepharose Fast Flow column (10 mL) equilibrated in 0.1 M potassium phosphate, pH 7.5. The column was washed with the equilibration buffer, and the prothrombin was eluted with a 0.1−0.7 M potassium phosphate, pH 7.5, gradient. A sample from each fraction was activated by *E. carinatus* venom and assayed for amidolytic activity. The peak fractions were pooled and dialyzed against 0.02 M HEPES and 0.1 M NaCl, pH 8.0. The dialysate was then concentrated using a stirred cell with a PM30 membrane (Amicon). The prothrombin was processed to thrombin by *E. carinatus* venom that had been cleared of components that adsorb to Amberlite CG50 cation-exchange resin. Following activation, the resulting thrombin was immediately loaded onto an Amberlite CG50 column (8 mL) equilibrated in 0.02 M HEPES and 0.1 M NaCl, pH 8.0. The column was washed with equilibration buffer and eluted with a 0.10−1.0 M NaCl gradient containing 20 mM HEPES, pH 8.0. The fractions were assayed for amidolytic activity, pooled, and concentrated. The purity of each mutant was assessed by SDS−PAGE and silver staining, and the thrombin concentration was determined by thrombin ELISA.

Kinetic Analysis of Fibrinopeptide A Release. Fibrinopeptide A (FPA) release was carried out as previously described (Higgins et al., 1983). Briefly, 500 μL reactions of fibrinogen and mutant thrombin in reaction buffer containing 137 mM NaCl, 2.5 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 9.47 mM sodium phosphate, pH 7.4, and 0.001% PEG-6000 were incubated at 37 °C and stopped with 43 μL of 3 M $HClO_4$. The precipitated protein was spun down in a microcentrifuge at 15 000 rpm for 5 min. The supernatant was transferred to an automatic sample injector for injection of 250 μL into the HPLC system. FPA and FPB were separated on a C_{18} column using a 15 min gradient of 22.5%−49.5% CH_3CN in 0.1% TFA. FPA and FPB eluted at 30.6% and 32.4% CH_3CN, respectively. For determination of EC_{50} (the concentration of thrombin causing the release of 50% of FPA after 60 min at 37 °C), mutant thrombin concentration was varied from 0.5 to 30 nM, and the reactions were incubated for 60 min at 37 °C and stopped. The data were fitted to the empirical function $P = P_{max}(1 - e^{-(\ln 2)[IIa]/EC_{50}})$, which best fit the data, using nonlinear regression to calculate the EC_{50} and associated standard error. P is the concentration of FPA released with a thrombin concentration of [IIa], and P_{max} is the maximum concentration of FPA that can be released.

For determination of the catalytic efficiency (k_{cat}/K_m) of FPA release, a thrombin concentration that allowed the complete release of both FPA and FPB in 60 min was used. The kinetic parameters were determined by the analysis of differential progress curves (Graycar & Estell, 1987) generated at two fibrinogen concentrations, 1.4 and 21 μM, from 0 to 60 min and from 0 to 70 min, respectively.

Briefly, for the simple one-substrate Michaelis−Menten mechanism with product inhibition, the differential rate equation is

$$v = \frac{dP}{dt} = \frac{V_m S}{K_m\left(1 + \frac{(S_0 - S)}{K_i}\right) + S} \quad (1)$$

where S is the substrate concentration at time t, S_0 the substrate concentration at time $t = 0$, $(S_0 - S)$ the product concentration, and K_i the product inhibition binding constant. Equation 1 can be rearranged to give a linear plot of $1/v$ versus $1/S$:

$$\frac{1}{v} = \frac{K_m(1 + (S_0/K_i))}{V_m}\frac{1}{S} + \frac{1 - (K_m/K_i)}{V_m} \quad (2)$$

In the absence of product inhibition, K_i approaches infinity and eq 2 simplifies to the Lineweaver−Burk equation. Performing a second progress curve using the same total enzyme but a new initial substrate level such that $S_0' = aS_0$ allows for the K_i to be determined. The ratio of the slopes from eq 2 for each reaction gives

$$\frac{m'}{m} = \frac{1 + aS_0/K_i}{1 + S_0/K_i} \quad (3)$$

Rearranging eq 3 we have

$$K_i = \frac{(a - m'/m)S_0}{(m'/m - 1)} \quad (4)$$

Using the derived K_i and the slope m and y-intercept b from one of the two progress curves, the K_m and V_m are calculated as follows:

$$K_m = \frac{mK_i}{b(K_i + S_0 + m/b)} \quad (5)$$

$$V_m = \frac{K_m(1 + S_0/K_i)}{m} \quad (6)$$

The progress curve of FPA release can be expressed as the integrated form of the Michaelis−Menten equation:

$$t = k_1 \ln\left(\frac{[A\alpha]_0}{[A\alpha]_0 - [FPA]}\right) + k_2[FPA] \quad (7)$$

where [FPA] is the concentratio of FPA at time t, $[A\alpha]_0$ is the initial concentration of Aα-chains of fibrinogen, k_1 is a constant, function of $[A\alpha]_0$, V_m, K_m, and K_i (the product inhibition constant), and k_2 is another constant, function of V_m, K_m, and K_i. $[A\alpha]_0$, k_1, and k_2 were determined by fitting eq 7 to the data. The inverse rate $1/v$ of FPA release at each time point was then calculated using the equation:

$$\frac{1}{v} = \frac{dt}{d[FPA]} = \frac{k_1}{([A\alpha]_0 - [FPA])} + k_2 \quad (8)$$

$1/v$ was plotted versus $1/[A\alpha] = 1/([A\alpha]_0 - [FPA])$, and the slope m and y-intercept b were then determined by curve fitting to a line. K_i, K_m, and V_{max} were subsequently calculated according to eqs 4−6.

Kinetic Analysis of Protein C Activation. Cell lysates containing recombinant human TM were prepared as previously described (Tsiang et al., 1995). The concentration of thrombomodulin was calculated by direct equilibrium binding of ^{125}I-DIP-thrombin to cells (Tsiang et al., 1990). Activation of PC was carried out in 50 mM Tris-HCl, pH 8.0, 2 mM $CaCl_2$, 100 mM NaCl, and 0.1% BSA. The aPC generated was assayed by hydrolysis of chromogenic substrate S-2366. To determine the affinity of mutant thrombin for TM, TM and PC were used at 0.5 nM and 1.0 μM, respectively, with increasing concentrations of mutant thrombin (0.1−20 nM). The K_d for TM binding and the rate of PC activation at saturating thrombin concentration (V_S) were determined by fitting the data to the binding equation $[EL] = [E_0][L]/(K_d + [L])$, where [EL] is the concentration of the thrombin/TM complex measured as the rate of PC activation and $[E_0]$ is the concentration of TM measured as V_S: the rate of PC activation when TM is saturated with thrombin and the concentration of PC is 1 μM. The background rate of PC activation in the absence of TM was subtracted.

To determine the catalytic efficiency (k_{cat}/K_m) of PC activation, steady-state kinetic conditions were used, with TM at 0.5 nM, mutant thrombin at the saturating concentration of 30 nM, and seven concentrations of PC (0.50, 0.66, 1.00, 1.30, 2.00, 4.00, and 10.00 μM). The initial velocity of PC activation for each concentration of PC was determined from a time course with time points taken at 7-min intervals. The K_m and k_{cat} for PC activation were again determined by fitting the Michaelis−Menten equation to the data assuming that the enzyme concentration was 0.5 nM, equal to the concentration of TM.

Platelet Aggregation. Platelet aggregation by recombinant wild-type and mutant thrombins (WT, E229A, E229K, and E229W) was performed with fresh citrated human platelet-rich plasma (PRP) using a Chrono-Log dual channel aggregometer (model 560-VS, Chrono-Log Corp., Havertown, PA). Recombinant human thrombin (WT or mutants) was added to 400 μL of prewarmed PRP (37 °C for 2 min) to a final concentration of 0.19−1.29 nM (WT), 2.7−22.0 nM (E229A), 5.9−29.7 nM (E229K), or 12.9−49.7 nM (E229W). The platelet aggregation stimulated by addition of wild-type or mutant thrombins was monitored by the aggregometer for 5 min as an increase in light transmission. The extent of platelet aggregation was quantitated by measuring the area under the tracing of the aggregation curve from 0 to 1 min after the addition of thrombin. The extent of aggregation was expressed in the arbitrary unit of cm^2 and plotted against the concentration of the agonist. The data were fitted to the empirical function which best fit the data: $A = A_{max}/(1 + e^{n(EC_{50} - [IIa])})$, where A is the extent of platelet aggregation at a thrombin concentration of [IIa], A_{max} is the maximum extent of platelet aggregation, and n is a parameter that controls the steepness of the logistic curve. The EC_{50} value (concentration of thrombin required to induce half-maximal

aggregation after 1 min) and associated standard error were determined by nonlinear regression.

Inhibition of Selected Thrombin Mutants by Antithrombin III in the Absence of Heparin. Selected purified thrombin mutants were screened for resistance to ATIII by determining the concentration required for half-maximal inhibition (IC_{50}) over a 30 min time period in the absence of heparin. Wild-type or mutant thrombins (5−80 nM) were incubated in selection buffer (20 mM Tris−acetate, pH 7.5, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$) plus 0.1% BSA with increasing concentrations (0.008−8.0 μM) of purified ATIII at 25 °C. After 30 min the inhibition reaction was terminated by the addition of 100 μM chromogenic peptide substrate S-2238. The residual activity due to uninhibited thrombin was determined by monitoring the hydrolysis of S-2238. IC_{50} values (ATIII concentration required for 50% inhibition of thrombin after 30 min) and the associated standard errors were determined by fitting the data to the empirical function: $A = 100(IC_{50})^n/((IC_{50})^n + [ATIII]^n)$, which best fit the data, using nonlinear regression, where A is the percent thrombin activity remaining at an antithrombin III concentration of [ATIII] and n is a parameter that controls the steepness of the curve.

Second-order rate constants for inhibition of purified thrombin mutants were determined as described previously (Ye et al., 1994). Thrombin (20−60 nM) was incubated in selection buffer plus 0.1% BSA with increasing concentrations (0.2−6.0 μM) of purified ATIII at 25 °C. At various time intervals (1−95 min) aliquots of the reaction mixture were diluted 10-fold into 100 μM S-2238 to terminate the inhibition reaction. The residual activity due to uninhibited thrombin was determined by monitoring the rate of hydrolysis of S-2238. Under pseudo-first-order conditions the time course of inhibition followed eq 9:

$$E_t = E_0 e^{-k't} \tag{9}$$

where E_0 is the thrombin activity at time $t = 0$ in A_{405}/s, E_t the thrombin activity at time t, and k' the observed pseudo-first-order rate constant. The pseudo-first-order rate constant of the decrease in activity due to inhibition by ATIII (k') was determined by nonlinear regression, and the second-order rate constant of inhibition (k_2) was determined from the slope of plots of k' versus ATIII concentration.

Heparin-Dependent Inhibition by Antithrombin III. The reaction buffer contained 20 mM Tris−acetate, pH 7.5, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.1% PEG-8000. Inhibition by ATIII in the presence of heparin was performed in the presence of competing substrate (S-2238) as previously described (Sheehan et al., 1993). Briefly, 150 nM ATIII, 1.0 unit/mL heparin, and 150 μM S-2238 were premixed in 590 μL of reaction buffer. This mixture was added to a spectrophotometric cuvette containing 10 μL of 300 nM mutant thrombin (final concentration of 5 nM), and hydrolysis of S-2238 was immediately measured for 2 min. The pseudo-first-order rate constant of inhibition (k') was determined by fitting eq 9 to the first derivative of the time course of S-2238 hydrolysis, which was directly calculated from the data. To determine the second-order rate constant k_2, k' was corrected for substrate competition and divided by the inhibitor concentration [I] as shown in eq 10:

$$k_2 = \frac{k'(1 + [S]/K_m)}{[I]} \tag{10}$$

where [S] is the substrate concentration during the reaction and K_m the Michaelis constant of the substrate for each mutant thrombin.

Clearance of Thrombin Activity from Human Plasma in Vitro. Thrombin and thrombin mutants (100 nM) were mixed with 10 volumes of fibrinogen-deficient human plasma (George King Biomedical) and incubated at 25 °C. At various time intervals aliquots of the reaction mixture were diluted 50-fold into 100 μM S-2238 to terminate the inhibition reaction. The residual activity due to uninhibited thrombin was determined by monitoring the hydrolysis of S-2238. The kinetics of the clearance of thrombin activity versus time approximately corresponded to a first-order exponential decay (eq 9). The half-life of thrombin and thrombin variants in human plasma was estimated using nonlinear regression analysis.

Anticoagulation Studies in Cynomolgus Monkeys. A cell-line stably expressing E229K prothrombin was constructed by transfecting CHO cell-line AA8 with pRc/CMV-hPT containing the E229K mutation. E229K prothrombin was secreted into CHO serum-free medium (Gibco), harvested, processed to thrombin, and purified as described previously (Gibbs et al., 1995).

All animals received humane care consistent with published NIH guidelines (NIH, 1985). Four adult Cynomolgus monkeys received 10 min intravenous infusions of purified E229K at a dose of 0.5 μg kg^{-1} min^{-1}, formulated in a total volume of 10 mL steric isotonic saline. Blood samples were drawn at intervals before, during, and after (up to 180 min) infusion to allow the coagulation state of the animal to be monitored. Plasma samples for determination of clotting times, [fibrinogen], [factor V], and [factor VIII], were anticoagulated with 0.38% citrate. Samples for measurement of aPC levels were anticoagulated with 0.38% citrate plus 25 mM *p*-aminobenzamidine. Samples before ($t = 0$) and after infusion ($t = 10$ min) for determination of fibrinopeptide A and D-dimer levels were anticoagulated with an inhibitor cocktail (containing citrate, heparin, and specific protease inhibitors) provided with the assay kit (Asserachrom FPA). Before ($t = 0$) and after ($t = 180$ min) samples for determination of platelet factor 4 and β-thromboglobulin were anticoagulated with 0.38% citrate plus adenosine, theophylline, and dipyridamole. Plasma was obtained from whole blood immediately. Whole blood samples for determination of platelet counts (before and after) were collected in 0.15% EDTA.

All clotting assays and quantitative determinations performed on plasma samples have been described previously (Gibbs et al., 1995). Template bleeding times were performed before infusion ($t = 0$) and at the peak of anticoagulation ($t = 20$ min). A blood pressure cuff was inflated to 40 mmHg, and a defined incision was made on the volar surface of the forearm using a commercial device (Surgicutt Jr.). The wound was blotted at 15 s intervals, and the time to the cessation of bleeding was recorded.

RESULTS

Initial Screening of 76 Saturation Mutants of Residues W50, K52, E229, and R233. Each mutant was transiently expressed in COS-7 cells and assayed for TM-dependent PC

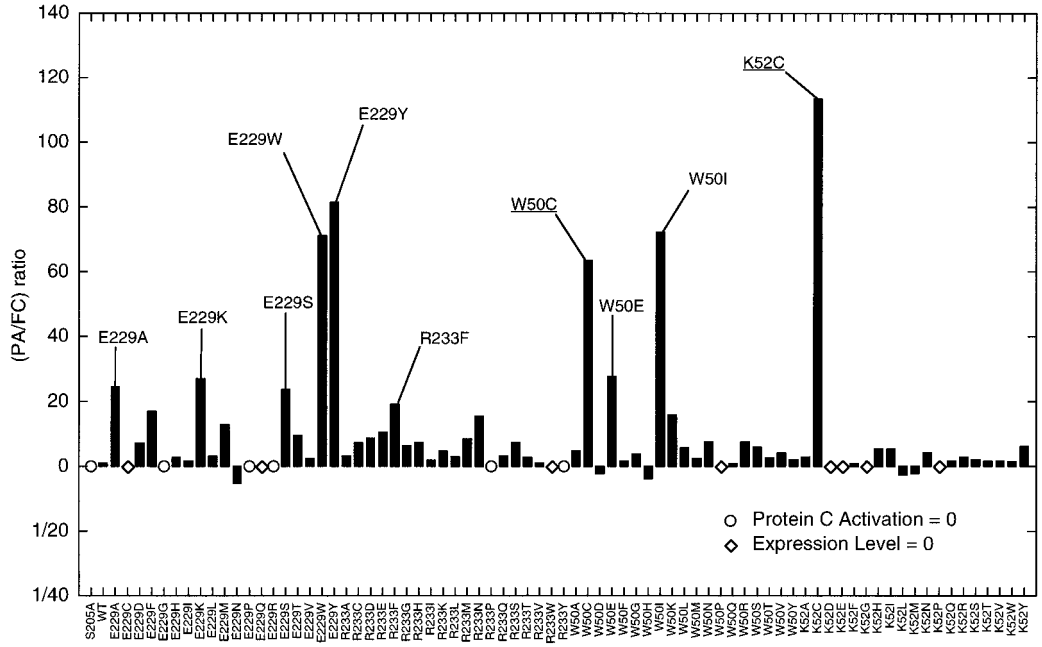

FIGURE 1: Initial screening of thrombin mutants for enhanced specificity toward protein C following saturation mutagenesis of residues W50, K52, E229, R233. PC activation (PA) and fibrinogen clotting (FC) specific activity was determined for each thrombin mutant in concentrated conditioned cell culture medium following transient transfection of COS-7 cells. Specificity was expressed as the PA/FC ratio where wild-type thrombin has a PA/FC ratio of 1. A ratio of greater than 1 indicates a greater specificity toward PC as compared to wild type (bars pointing upward), and a ratio of less than 1 indicates a lower specificity toward PC (bars pointing downward). The mean relative standard error for the PA/FC ratio of all mutants is 40%.

Table 1: Secondary Screening of Thrombin Mutants with Enhanced Protein C Activation Activity Compared to Fibrinogen Clotting Activity[a]

thrombin mutants[b]	FPA release, EC_{50} (nM)	TM binding, K_d (nM)	PC activation,[c] V_S (pmol of aPC/h)	ATIII inhibition, IC_{50} (μM)
WT	ND	3.46 ± 0.41	6.12 ± 0.59	0.069 ± 0.003
E229A	0.69 ± 0.05	3.75 ± 1.13	2.37 ± 0.66	1.97 ± 0.23
E229K	2.90 ± 0.16	4.38 ± 0.67	1.38 ± 0.16	5.73 ± 0.41
E229S	0.75 ± 0.02	4.69 ± 0.70	2.54 ± 0.30	1.34 ± 0.09
E229W	3.55 ± 0.30	3.43 ± 0.40	1.36 ± 0.11	0.78 ± 0.03
E229Y	0.34 ± 0.01	4.12 ± 0.66	1.80 ± 0.22	1.08 ± 0.07
R233F	0.49 ± 0.01	3.52 ± 0.46	0.74 ± 0.07	1.48 ± 0.11
W50E	0.35 ± 0.02	1.88 ± 0.47	0.31 ± 0.05	5.28 ± 0.10
W50I	0.125 ± 0.016	3.32 ± 0.44	1.36 ± 0.13	1.53 ± 0.06

[a] Mutants that displayed a PA/FC ratio >18 following initial screening (Figure 1) were purified and characterized in more detail. [b] Errors represent standard errors. [c] V_S = rate of PC activation for 0.5 nM TM saturated with thrombin and 1 μM PC.

activation and fibrinogen clotting activity. Specificity for protein C was expressed as the ratio of the specific activity of PC activation (PA) over the specific activity of fibrinogen clotting (FC) (Figure 1). The ratio for wild-type thrombin was assigned a value of 1. Nine mutants with a PA/FC ratio of greater than 18, E229K, E229S, E229W, E229Y, R233F, W50C, W50E, W50I, and K52C, were identified in addition to E229A thrombin, the prototype protein C activator. Because the PA/FC ratios for mutants with very low specific fibrinogen clotting activity (FC) values may be subject to error, all these mutants except W50C and K52C were selected for secondary screening. W50C and K52C were not selected for further analysis despite their favorable PA/FC ratio because of their free cysteine, which has the potential to cause dimer formation or disruption of normal disulfide bridges within the thrombin molecule.

Secondary Screening of 7 Mutants with an Enhanced PA/FC Ratio. The seven thrombin mutants with a PA/FC ratio >18 identified in the initial screening were subcloned into the Sindbis virus expression vector for medium scale expression. About 100−120 μg of each mutant thrombin was purified for kinetic characterization. For secondary screening, the EC_{50} for FPA release, the K_d for TM binding, the V_S for PC activation, and the IC_{50} of ATIII inhibition were determined (Table 1). The affinity for TM of all the mutants tested did not change significantly from that of the wild type. E229K and E229W thrombins were distinguished by their decreased activity toward fibrinogen, displaying a 4−5-fold increase in the EC_{50} for FPA release compared to E229A thrombin. However, their V_S for TM-dependent PC activation remained comparable to that of E229A thrombin. In addition, E229K but not E229W thrombin displayed increased resistance to ATIII. These two mutants were subjected to more detailed kinetic characterization to determine which had superior specificity for PC.

Final Discrimination between E229K and E229W Thrombins. The kinetic parameters of FPA release and PC activation were determined for wild-type, E229A, E229K, and E229W thrombins (Table 2). The k_{cat}/K_m of FPA release for E229K and E229W were almost identical and ~5-fold lower than that of E229A and ~300-fold lower than that of wild-type thrombin. The k_{cat}/K_m of PC activation in the presence of TM for E229K did not change from that of E229A while that of E229W decreased by 50.5% with respect to E229A. The net result is that E229K had a 130.9-fold higher specificity for PC over fibrinogen than wild type,

Table 2: Kinetic Analysis of Purified Thrombin Mutants with Enhanced Specificity for Protein C over Fibrinogen

thrombin mutants[a]	fibrinopeptide A release[b]			protein C activation[c]			specificity ratio[d]
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}\cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}\cdot s^{-1}$)	CE_{PA}/CE_{FR}
WT	59 ± 13	3.16 ± 0.38	18.7 ± 6.7	0.650 ± 0.056	3.19 ± 0.51	0.20 ± 0.05	0.0107 (1)
E229A	7.88 ± 0.03	23.6 ± 0.1	0.334 ± 0.003	0.189 ± 0.005	2.00 ± 0.13	0.095 ± 0.009	0.2844 (26.59)
E229K	2.53 ± 0.23	38 ± 3	0.07 ± 0.01	0.21 ± 0.01	2.15 ± 0.26	0.098 ± 0.016	1.4 (130.90)
E229W	3.12 ± 0.02	52.8 ± 0.3	0.059 ± 0.001	0.148 ± 0.004	3.12 ± 0.19	0.047 ± 0.004	0.7966 (74.48)

[a] Errors are standard errors. [b] The thrombin concentrations used for WT, E229A, E229K, and E229W were 0.3, 7.0, 30, and 25 nM, respectively. [c] Thrombin concentration was 30 nM, TM was 0.5 nM, and PC was varied from 0.5 to 10 μM. For calculation of k_{cat} the enzyme concentration was assumed to be equal to that of TM (0.5 nM) saturated with thrombin. [d] The specificity ratio is the ratio of the catalytic efficiency for protein C activation (CE_{PA}) divided by the catalytic efficiency for fibrinopeptide A release (CE_{FR}). The relative specificity compared to that of wild-type thrombin (specificity $ratio_{mutant}$/specificity $ratio_{wild\ type}$) is in parentheses.

Table 3: Further Characterization of Thrombin Mutant E229K

thrombin mutants[a]	platelet aggregation, EC_{50} (nM)	S-2238 hydrolysis, K_m (μM)	ATIII/heparin inhibition, $k_2 \times 10^{-8}$ ($M^{-1}\cdot min^{-1}$)	ATIII inhibition, $k_2 \times 10^{-5}$ ($M^{-1}\cdot min^{-1}$)	plasma inactivation, $t_{1/2}$ (s)
WT	1.3 ± 0.9	2.95 ± 0.46	5.98 ± 0.14	3.25 ± 0.18	36 ± 9
E229A	15 ± 7	50.2 ± 2.7	0.63 ± 0.01	0.47 ± 0.04	169 ± 48
E229K	23 ± 9	63.2 ± 4.9	0.181 ± 0.005	0.150 ± 0.004	934 ± 224

[a] Errors represent standard errors.

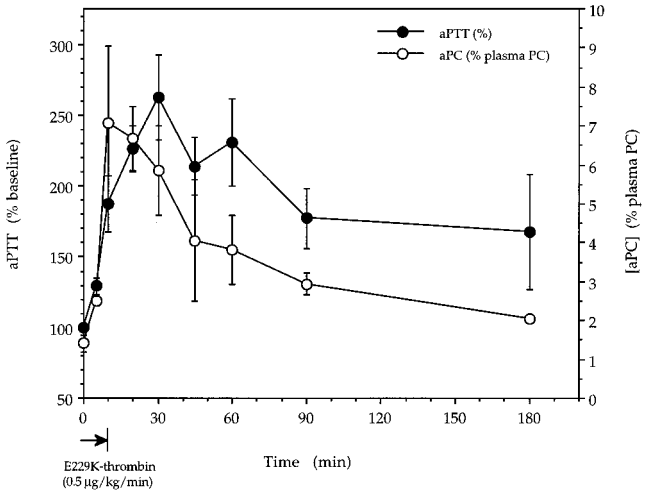

FIGURE 2: Activation of endogenous protein C and anticoagulation following infusion of E229K thrombin in Cynomolgus monkeys: E229K thrombin was infused at a dose of 0.5 μg kg^{-1} min^{-1} for 10 min ($n = 4$). Anticoagulation was monitored by prolongation of the aPTT (baseline = 19.8 s); endogenous aPC levels were recorded as a percent of total monkey plasma PC. Error bars represent standard errors.

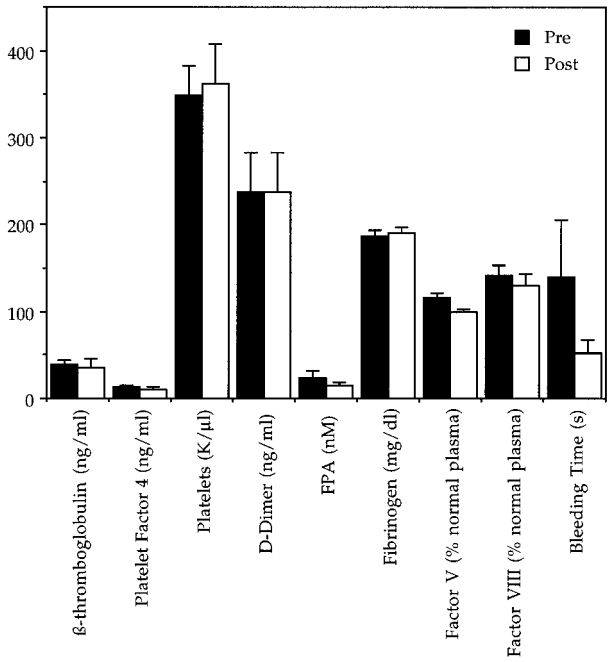

FIGURE 3: Maintanence of hemostatic parameters following infusion of E229K thrombin in Cynomolgus monkeys: Preinfusion (Pre, $t = 0$) and postinfusion levels (Post) of hemostatic parameters were determined following 10 min infusions of E229K thrombin at a dose of 0.5 μg kg^{-1} min^{-1} ($n = 4$). Postinfusion values were determined at $t = 180$ min except for D-dimer and FPA ($t = 10$ min) and bleeding time ($t = 20$ min). Error bars represent standard errors.

whereas E229W had only a 74.48-fold higher specificity for PC. E229K thrombin was also ~18-fold less efficient than wild type in its ability to activate platelets (Table 3) and also displayed increased resistance to antithrombin III compared to wild type, in both the presence and absence of heparin, by a factor of 33 and 22, respectively. The increased resistance to antithrombin III may be correlated to the observed 26-fold increase in plasma half-life *in vitro* (Table 3).

Anticoagulation in Vivo following Infusion of E229K Thrombin. Infusion of E229K thrombin in four Cynomolgus monkeys at a dose of 0.5 μg kg^{-1} min^{-1} for 10 min caused activation of endogenous protein C and an anticoagulant effect as measured by prolongation of the aPTT (Figure 2). The peak anticoagulant effect was observed between $t = 10$ min and $t = 30$ min over which period the aPTT was prolonged by approximately 2.3-fold and approximately 6.6% of total plasma PC was observed to be in the aPC form. The effect of the infusion was reversible with aPC levels returning to baseline with a half-life of approximately 60 min. Notably, template bleeding times measured at the peak of anticoagulation were not prolonged (Figure 3).

Fibrinogen was not consumed and FPA and D-dimer levels were not elevated following infusion of E229K thrombin (Figure 3), indicating that E229K thrombin did not cause detectable fibrinogen clotting *in vivo*. Similarly, the levels of coagulation factors V and VIII were not perturbed (Figure 3), indicating that E229K thrombin did not activate the coagulation cascade. In addition, platelets were not consumed and plasma levels of platelet secretion granule components, β-thromboglobulin and platelet factor 4, were not elevated (Figure 3), indicating that E229K thrombin did not stimulate platelet activation *in vivo*.

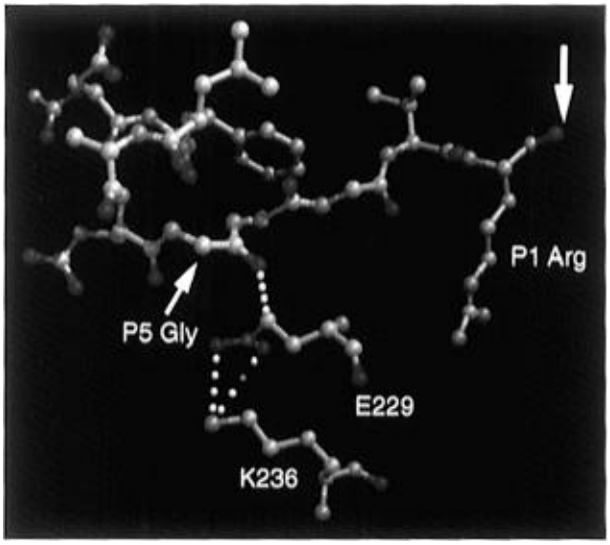

FIGURE 4: Specific contacts of the side chain of thrombin residue E229 in the complex of thrombin with FPA. The image was constructed using MidasPlus (UCSF) on the basis of the crystal structure of human thrombin complexed with FPA (Stubbs et al., 1992). Fibrinopeptide A is shown with the P1 and P5 residues highlighted. The carbonyl oxygen of the amide bond in FPA cleaved by thrombin is indicated with an arrow. The only thrombin residues shown are E229 and K236. E229 is in van der Waals contact with the glycine residue at P5 in FPA (E229 Cγ−P5 Gly O distance = 3.43 Å) and forms a salt bridge with K236 (E229 Oϵ1−K236 Nζ = 4.11 Å; E229 Oϵ2−K236 Nζ = 3.33 Å). Atom coloring is as follows: carbon = brown, oxygen = red, and nitrogen = blue. Interatomic contacts with the side chain of E229 are indicated by white dotted lines.

DISCUSSION

In previous *in vivo* studies where thrombin infusion has been utilized to achieve an anticoagulant effect through the activation of endogenous PC, wild-type thrombin displayed a weak anticoagulant activity *in vivo* due to rapid clearance by ATIII and the dose-limiting effects of the familiar procoagulant functions of wild-type thrombin (fibrinogen clotting and platelet activation) (Hanson et al., 1993; Gibbs et al., 1995). In this study, screening of a library of 76 thrombin mutants, where all possible substitutions at positions W50, K52, E229, and R233 were sampled, identified a single amino acid substitution, E229K, that conferred optimal anticoagulant activity. Substitution of thrombin residue E229 with lysine resulted in a 267-fold decrease in the catalytic efficiency of cleavage of the fibrinogen Aα chain, an 18-fold decrease in the efficiency of cleavage of the platelet thrombin receptor, and a 33- and 22-fold decrease in the rate of inhibition by ATIII in the presence and absence of heparin. In contrast, the affinity for TM was unchanged and the catalytic efficiency of TM-dependent PC activation was decreased by only 51%.

Thrombin residue E229 is located at surface of thrombin on the edge of the active site cleft where it is well positioned to interact with substrates and active site inhibitors such as ATIII on the N-terminal side of the scissile bond. In the crystal structure of human thrombin complexed with FPA, E229 is observed in van der Waals contact with a glycine residue at P5 in FPA (Figure 4). As discussed previously (Gibbs et al., 1995), the most attractive structural rationalization for the differential effects of the substitution of E229 on substrate recognition is the hypothesis that PC binds thrombin in a mode that is distinct from the other substrates and less affected by the replacement of E229. This hypothesis gains some support from the crystal structure of human thrombin complexed with FPA, where FPA is bound in a hooked conformation (Figure 4) due to a type II β-turn that requires the residue at P5 to be glycine to avoid strain (Stubbs et al., 1992). In PC, the absence of glycine at P5 or elsewhere proximal to the cleavage site suggests PC is likely to bind thrombin in an extended conformation unlike that observed with FPA.

On average, the catalytic deficiencies toward the procoagulant substrates and inhibition by ATIII are approximately 5-fold more severe for the E229K variant than for E229A. This may be due to the introduction of a residue of opposite charge or altered conformation at position 229, leading to less favorable interactions with these substrates. Alternatively, the effects of the lysine substitution may be more indirect. In thrombin, the side chain of E229 can form an ion pair with the side chain of K236 (distance between charged centers = 3.7 Å) (Figure 4) (Bode et al., 1992). The introduction of another lysine residue at position 229 may cause charge repulsion and more generalized structural changes that prevent optimal interactions with the procoagulant substrates.

The effects of the improved specificity and resistance to clearance of E229K thrombin observed *in vitro* were also manifested *in vivo* upon infusion of E229K thrombin in Cynomolgus monkeys. E229K thrombin was a more potent PC activator and anticoagulant than E229A thrombin in terms of both magnitude and duration of the effects. Previously, following infusion of E229A thrombin at a dose of 2.5 μg kg^{-1} min^{-1} the maximal prolongation of the aPTT was 2.3-fold; aPC levels peaked at 5.5% of total plasma PC and returned to baseline with a half-life of approximately 15 min (Gibbs et al., 1995). Infusion of E229K thrombin caused effects of a similar magnitude (2.6-fold maximal prolongation of aPTT and peak conversion of 7.1% of plasma PC to aPC) but at a 5-fold lower dose of 0.5 μg kg^{-1} min^{-1}. In addition, aPC levels returned to baseline more slowly with a half-life of approximately 60 min, which results in a cumulative increase in potency of greater than 10-fold when the area under the decay curve is taken into account. Although it is difficult to extrapolate *in vitro* clearance studies to *in vivo* situations, the increased potency of E229K thrombin observed *in vivo* may be correlated to its prolonged half-life in plasma *in vitro* (Table 3) and its resistance to purified ATIII (Table 3), which is the most important physiological inhibitor of thrombin (Rosenberg, 1987; Olsen & Björk, 1992).

The enhanced specificity of E229K thrombin toward PC was also manifested *in vivo*. Previous studies in baboons (Hanson et al., 1993) and Cynomolgus monkeys (Gibbs et al., 1995) demonstrated that, following infusion of wild-type thrombin, the effects of both the procoagulant and the anticoagulant functions of thrombin were evident. In addition to activating PC and prolonging clotting times, wild-type thrombin also cleaved fibrinogen (fibrinogen consumption, elevation of FPA and D-dimer), activated the coagulation cascade (consumption of factor VIII and factor V), and activated platelets (elevation of platelet factor 4 and β-thromboglobulin). Following infusion of the prototype PC activator, E229A thrombin, in monkeys (Gibbs et al., 1995), the anticoagulant effect was observed in the absence of almost all of the aforementioned procoagulant side effects with the only indication of any procoagulant activity being a mild elevation of FPA levels (42 nM compared with 254 nM for

wild type). In the present study there was no indication of any residual procoagulant function following infusion of E229K thrombin. FPA levels remained constant at preinfusion levels (15 nM), which may reflect the enhanced specificity of E229K thrombin for PC over fibrinogen.

A notable feature of the anticoagulation studies in Cynomolgus monkeys is that anticoagulation by E229K thrombin as well as E229A thrombin (Gibbs et al., 1995) was not associated with prolonged template bleeding times even at elevated doses where the aPTT was in excess of 200 s (>10-fold prolonged, $n = 1$) (data not shown). The mechanism of anticoagulation mediated by thrombin variants is through endogeneous aPC generation which by inactivating factors Va and VIIIa inhibits generation of factor Xa and thrombin. This mechanism is similar to that shared by inhibitors acting directly on coagulation factors upstream of thrombin. In contrast to direct thrombin inhibitors [PPACK (Hanson & Harker, 1988) and hirudin (Kelly et al., 1991)] and direct antiplatelet agents [mAbs versus vWF and GPIIbIIIa (Cadroy et al., 1994) and RGDV peptide (Cadroy et al., 1989)], anticoagulants that act by inhibiting thrombin generation [tick anticoagulant peptide (Sitko et al., 1992) and aPC (Gruber et al., 1990, 1991)] have shown antithrombotic efficacy without perturbing primary hemostasis as measured by bleeding time.

Therefore, E229K thrombin which can function as a potent anticoagulant *in vivo* without any procoagulant side effects and without prolonging bleeding time may be a superior antithrombotic agent with reduced potential for bleeding complications.

ACKNOWLEDGMENT

We thank Pam Mack, DVM (Tulane Regional Primate Research Center) for assistance with animal studies and Terry Terhorst for the synthesis of the oligonucleotides used in this study.

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BI9616108