Ecotin Is a Potent Anticoagulant and Reversible Tight-Binding Inhibitor of Factor Xa

Jana L. Seymour,[‡] Robert N. Lindquist,[‡,§] Mark S. Dennis,[‡] Barbara Moffat,[∥] Daniel Yansura,[⊥] Dorothea Reilly,[#] Mary E. Wessinger,[▽] and Robert A. Lazarus*[,‡]

Departments of Protein Engineering, Protein Chemistry, Cell Genetics, Fermentation Research and Development, and Cardiovascular Research and Development, Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, California 94080

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ABSTRACT: Ecotin, a serine protease inhibitor found in the periplasm of *Escherichia coli*, has been characterized as an extremely potent anticoagulant and reversible tight-binding inhibitor of human factor Xa (FXa). The ecotin gene was cloned by PCR, highly expressed in *E. coli*, and purified from the *E. coli* periplasm. The binding of ecotin to FXa was stoichiometric with an equilibrium dissociation constant K_i of 54 pM. The association rate constant was 1.35×10^6 M^{-1} s^{-1}, and the dissociation rate constant, measured in the presence of human leukocyte elastase (HLE) to prevent reassociation of ecotin with FXa, was 6.5×10^{-5} s^{-1}. Ecotin prolonged clotting time ca. 10-fold at 0.3 μM and at 2 μM in activated partial thromboplastin time and prothrombin time assays, respectively. Ecotin did not effectively inhibit the human plasma proteases thrombin, tissue factor·factor VIIa, factor XIa, activated protein C, plasmin, or tissue plasminogen activator (t-PA); however, it did potently inhibit factor XIIa, plasma kallikrein, HLE, and bovine trypsin and chymotrypsin. Coincubation of ecotin and FXa at 10 μM each resulted in a $(\text{ecotin})_2 \cdot (\text{FXa})_2$ complex as determined by gel filtration. Dimerization of ecotin alone was measured by fluorescence titration which yielded a K_d of ca. 390 nM. FXa cleaved ecotin slowly at pH 4.0 between M84 and M85. Replacement of the P_1 Met84 residue with Arg and Lys led to FXa inhibitors with K_i values of 11 and 21 pM, respectively. The P_1 Arg and Lys mutants also significantly inhibited thrombin, factor XIa, activated protein C, plasmin, factor XIIa, kallikrein, and bovine trypsin and chymotrypsin but did not inhibit tissue factor·factor VIIa, t-PA, or HLE.

Factor Xa (FXa)[1] is a vitamin K-dependent glycosylated serine protease that plays a fundamental role in the coagulation cascade and in maintaining hemostasis (Davie et al., 1991; Mann et al., 1992). It is produced from its zymogen factor X upon activation of either the intrinsic or extrinsic pathways of coagulation and, in the presence of factor Va, Ca^{2+}, and a suitable phospholipid surface, forms the prothrombinase complex. This complex generates thrombin which ultimately results in the formation of a stable fibrin clot. Since FXa is common to both coagulation pathways, anticoagulants that target inhibition of FXa may prevent fibrin-rich thrombus formation and have utility in the treatment of thrombotic diseases.

Protein inhibitors of proteases play critical roles in the regulation of proteolytic activity in a wide variety of physiological processes. They have been extensively studied from functional, structural, and mechanistic perspectives (Laskowski & Kato, 1980; Bode & Huber, 1992). Factor Xa is regulated by at least two different plasma protease inhibitors *in vivo*. Antithrombin III (ATIII) is a serpin that, in the presence of heparin or other glycosaminoglycans, gives rise to rapid and irreversible inhibition of FXa, thrombin, and other plasma proteases (Björk & Danielsson, 1986). The mechanism of heparin-mediated ATIII inhibition is thought to involve either a conformational change in ATIII or the formation of a ternary complex (Björk & Danielsson, 1986). Tissue factor pathway inhibitor (TFPI) is a protein containing three tandem Kunitz domains and is a slow tight-binding inhibitor of FXa; it also inhibits the tissue factor·factor VIIa (TF·FVIIa) complex in a FXa-dependent manner (Broze et al., 1990). On the basis of *in vitro* properties of TFPI, it is thought to regulate the tissue factor induced (extrinsic) coagulation pathway by a feedback mechanism (Broze, 1992).

Two potent inhibitors of FXa from exogenous sources have also been described. Antistasin is a potent 119-residue protein inhibitor of FXa found in the salivary glands of the Mexican leech *Haementaria officinalis* (Tuszynski et al., 1987). It is a reversible slow tight-binding inhibitor with an estimated dissociation constant between 0.31 and 0.62 nM (Dunwiddie et al., 1989). Tick anticoagulant peptide (TAP) is a 60-residue protein derived from the salivary glands of the tick *Ornithodoros moubata* that also reversibly and potently inhibits FXa (Waxman et al., 1990); dissociation constants of 0.18–0.59 nM have been reported (Jordan et al., 1990). Both antistasin and TAP prevent venous thrombosis in a rabbit model (Vlasuk et al., 1991).

Ecotin has been previously characterized as a periplasmic protein in *Escherichia coli* and an inhibitor of the pancreatic

* To whom correspondence should be addressed.
‡ Department of Protein Engineering.
§ On sabbatical leave from the Department of Chemistry and Biochemistry, San Francisco State University.
∥ Department of Protein Chemistry.
⊥ Department of Cell Genetics.
Department of Fermentation Research and Development.
▽ Department of Cardiovascular Research and Development.
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[1] Abbreviations: FXa, factor Xa; TF, tissue factor; TFPI, tissue factor pathway inhibitor; TAP, tick anticoagulant peptide; HLE, human leukocyte elastase; PCR, polymerase chain reaction; MUGB, 4-methylumbelliferyl *p*-guanidinobenzoate; TNCT buffer, 50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM $CaCl_2$, 0.005% Triton X-100; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PBS, phosphate buffered saline, SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; BAPA, N^{α}-benzoyl-L-arginine-*p*-nitroanilide; DEAE, diethylaminoethyl; PVDF, polyvinylidene difluoride; PT, prothrombin time; APTT, activated partial thromboplastin time.

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serine proteases trypsin and chymotrypsin (bovine), porcine elastase, and rat mast cell chymase. It does not inhibit any known proteases from *E. coli* and has been postulated to play a role in protecting the bacteria from exogenous proteases found in the mammalian gut (Chung et al., 1983; McGrath et al., 1991b). Ecotin has recently been crystallized alone (Shin et al., 1993) and as a complex with rat trypsin mutant D102N (McGrath et al., 1991a); the structure of the complex has recently been determined (McGrath et al., 1994). In this report, we show that ecotin is a potent anticoagulant and the most potent reversible FXa inhibitor described to date.

MATERIALS AND METHODS

Materials. Human factor Xa, activated protein C, thrombin, factor VIIa, and factor XIa were purchased from Haematologic Technologies, Inc. Bovine trypsin, bovine chymotrypsin, Triton X-100, 4-methylumbelliferyl *p*-guanidinobenzoate (MUGB), and CHAPS were from Sigma. Human leukocyte elastase (HLE) was from Elastin Products, Inc. Human plasmin was purchased from Kabi. Human factor XIIa and human plasma kallikrein were obtained from Enzyme Research Laboratories, Inc. Single chain tissue plasminogen activator (t-PA) was obtained from N. Paoni (Genentech). The substrates were obtained as follows: N^{α}-Benzoyl-L-arginine-*p*-nitroanilide (BAPA), Bachem; Spectrozyme fXa, American Diagnostica; S2366, S2302, and S2288, Chromogenix; S2251, Kabi; MeOSuc-Ala-Ala-Pro-Val-pNA, Calbiochem; Suc-Ala-Ala-Pro-Phe-pNA, Sigma. Recombinant human TF_{1-243} was produced in *E. coli* and purified as previously described (Paborsky et al., 1989). Polyacrylamide gels were purchased from Novex. DEAE fast flow and Superdex resins were from Pharmacia LKB Biotechnology, Inc. Oligonucleotides were synthesized using hydrogen phosphonate chemistry (Froehler et al., 1986) and purified by polyacrylamide gel electrophoresis (PAGE). Restriction enzymes were from New England Biolabs. Affi-Gel-15 was obtained from Bio-Rad. All other reagents were of the highest grade commercially available.

Cloning of the Ecotin Gene. All DNA manipulations were performed according to standard procedures unless otherwise indicated (Sambrook et al., 1989). The ecotin gene was cloned from *E. coli* chromosomal DNA using the polymerase chain reaction (PCR). Based on the published sequence of the ecotin gene (McGrath et al., 1991b; Lee et al., 1991), two primers were designed which were complementary to the 5′ and 3′ ends of the gene, and additionally encoded *Xba*I and *Bam*HI restriction sites. The primers had the following sequences: 5′-CTGGACTCTAGAATTATGAAGACCATTCTACCTGCAGTA and 5′-TCTGAGGATCCAGGCCTTTAGCGAACTACCGCGTTGTCAAT. The PCR reaction was carried out with Ampli-Taq polymerase on *E. coli* strain W3110 (ATCC 27325) chromosomal DNA using a Perkin-Elmer thermocycler under the following conditions: denaturation for 1 min at 94 °C, annealing for 2 min at 50 °C, and extension for 3 min at 72 °C. After 30 cycles the reaction was phenol/chloroform extracted, ethanol precipitated, and digested with *Xba*I and *Bam*HI. The digest was then electrophoresed on a 5% polyacrylamide gel in 89 mM Tris borate and 1 mM EDTA, pH 7.9; the gel was stained with ethidium bromide, and a band of approximately 500 base pairs was excised and eluted. The ecotin expression vector was created by ligating the fragment obtained from the PCR reaction into a derivative of phGH1 (Chang et al., 1987) from which the *Xba*I–*Bam*HI insert had been removed. Transformation of the ligation mixture into *E. coli* strain 27C7, a derivative of W3110, followed by restriction analysis and dideoxy sequencing (Sanger et al., 1977) led to pEt3, a plasmid encoding the correct ecotin sequence.

Expression and Purification of Recombinant Ecotin. Cultures of 27C7 retransformed with pEt3 were grown at 37 °C for 30 h in an aerated 10-L fermentor in medium containing 110 μg/mL ampicillin, 11 g/L yeast extract, 11 g/L casein hydrolysate, 16.4 mM K_2HPO_4, 9.2 mM NaH_2PO_4, 47.4 mM $(NH_4)_2SO_4$, 3.7 mM sodium citrate, 22.1 mM KCl, 7.7 mM $MgSO_4$, 110 μM $FeCl_3$, 15 μM $ZnSO_4$, 16.2 μM $CoCl_2$, 15.9 μM Na_2MoO_4, 17.6 μM $CuSO_4$, 17.7 μM H_3BO_3, and 18.2 μM $MnSO_4$. Glucose was added to maintain glucose excess or avoid anaerobiosis, depending on cell density, and the pH was maintained at pH 7.4 with the addition of NH_4OH. The cell density at harvest was 116 OD_{550}.

Recombinant ecotin was purified as follows. Frozen cell paste (400 g) was subjected to osmotic shock by thawing in 10 volumes of 20 mM Tris, 5 mM EDTA, pH 7.5 and stirring at 4 °C for 15 min. The cells were removed by centrifugation at 10800*g* for 45 min, and the supernatant was clarified by filtration through a 0.45-μm membrane. The periplasmic fraction obtained (3000 mL) was mixed with 500 mL of DEAE-Sepharose fast flow which had been previously equilibrated in 20 mM Tris and 5 mM EDTA, pH 7.5, and allowed to settle at 4 °C overnight. The supernatant containing the ecotin was then decanted and filtered through a 0.45-μm membrane. A portion of the supernatant (500 mL) was adjusted to 0.1 M NaCl and loaded onto 200 mL of silica adsorbant support (Davison Chemicals) equilibrated in phosphate buffered saline (PBS) at pH 7.4. The column was washed with PBS and eluted with 7.5 M urea, 50 mM Tris, and 5 mM glycine, pH 8.5. A fraction of the silica column eluate was adjusted to pH 3.0 with HCl and loaded onto a Vydac C4 reverse-phase HPLC column (10 × 250 mm) equilibrated with 0.1% TFA. The column was washed and then eluted with a linear gradient of 25–40% acetonitrile over 50 min. Following analysis by SDS–PAGE, fractions containing ecotin were pooled, and acetonitrile was evaporated under a stream of nitrogen. The resulting concentrated pool was subjected to size exclusion chromatography on a Superdex column (SX200, 26/60) equilibrated with 50 mM Tris, pH 7.5, at 6 °C, at a flow rate of 2 mL/min. Fractions containing ecotin from several such runs were pooled and passed over a Pharmacia Q-Sepharose HR column (16 × 100 mm) equilibrated in 50 mM Tris, pH 8.0, to reduce endotoxin levels; purified ecotin was stored at −80 °C.

General Activity Assays. Routine enzyme inhibition assays were conducted in a microtiter format, and absorbance changes were monitored on an SLT EAR340AT plate reader controlled by a Macintosh SE computer equipped with Biometallics DeltaSoftII software. Nonlinear regression analysis was carried out using KaleidaGraph v2.1.4 (Synergy Software).

Ecotin activity and quantitation was carried out using active site titrated trypsin (see below). Trypsin inhibition was measured as follows: 10 nM trypsin, 25 μL of 10× trypsin buffer (500 mM Tris, pH 8.0, 100 mM $CaCl_2$), and inhibitor were incubated for 15 min at room temperature in a total volume of 200 μL. The absorbance at 405 nm was recorded upon addition of 50 μL of 2.5 mM Spectrozyme fXa. Inhibition of FXa was measured in the same manner except the assay contained 5 nM human FXa, 25 μL of 10× TNCT buffer (500 mM Tris, pH 7.5, 1.5 M NaCl, 20 mM $CaCl_2$, 0.05% Triton X-100), and inhibitor.

Ecotin Mutants. The phagemid used for mutagenesis was made by subcloning the 505 base pair *Xba*I–*Bam*HI fragment

of pEt3, which encodes the ecotin gene and its native signal sequence, into phagemid pA4G32. The pA4G32 phagemid was originally constructed by subcloning the synthetic gene encoding the APPI sequence (Castro et al., 1990) in place of the human growth hormone sequence in the phagemid phGHam-g3 (Lowman et al., 1991). The pA4G32 phagemid also contains the alkaline phosphatase promoter, stII secretion signal (deleted in the ecotin subclone), the f1 and colE1 origins of replication, and the ampicillin resistance gene. Site-directed mutagenesis was used to incorporate Arg, Lys, Ala, Glu, or Asp at the Met84 P_1 position[2] (Kunkel, 1985); clones were confirmed by dideoxy sequence analysis (Sanger et al., 1977). Phagemids encoding the desired mutations were then transformed into *E. coli* strain 27C7 and checked for expression as follows. One-liter cultures of each mutant were grown for 20 h at 37 °C in low phosphate minimal media (Chang et al., 1987) containing 50 μg/mL carbenicillin. The cells were harvested by centrifugation and the periplasmic contents obtained by the osmotically shocking the resuspended cell pellets in 4 mL of 10 mM Tris, pH 8.0, and 1 mM EDTA. After being stirred for 1 h at 4 °C, the suspension was spun and the supernatant harvested. These samples were assayed for ecotin expression by trypsin inhibition assays (data not shown) and by SDS–PAGE (Figure 1).

Mutant ecotin proteins were purified from the periplasmic contents by binding to 2.5 mL of Affi-Gel-15–trypsin affinity resin prepared according to the manufacturer's directions. The resin was equilibrated and washed with 20 mM Tris, pH 7.5, and 5 mM $CaCl_2$, and mutants were eluted with 10 mM HCl. Fractions containing inhibitory activity were pooled and further purified by reverse-phase HPLC using a 10 × 250 mm 218TP510 Vydac C18 column (5 μm) monitored at 214 nm. After loading and washing in 0.1% TFA, a 15–60% gradient of CH_3CN containing 0.1% TFA (0.5%/min, 5 mL/min, 1 min/fraction) was used to elute the column; the ecotin mutants eluted at ca. 40% CH_3CN. Active fractions were vacuum evaporated to remove the CH_3CN, pooled, and stored at 4 °C.

Determination of Equilibrium Dissociation Constants. Apparent K_i values (K_i^*) for the inhibition of FXa by ecotin and mutants were measured as follows. Stock solutions of trypsin were quantitated by active site burst titration with MUGB as previously described (Jameson et al., 1973) using a Fluoroskan II plate reader at excitation wavelength of 355 nm and emission wavelength of 460 nm. Active site titrated trypsin was then used to quantitate ecotin active sites using BAPA as the substrate; a stoichiometry of 1:1 was assumed (Chung et al., 1983). Factor Xa was then quantitated by active site titration using ecotin as a standard, again assuming a 1:1 complex formation. There was good agreement with protein concentrations determined by amino acid analysis, Bradford analysis (Bradford, 1976), and active site titrations. Values of K_i^* were determined by incubation of various dilutions (0–8.7 nM final concentration) of the quantitated ecotin or ecotin mutant in TNCT buffer with human FXa (1–5 nM final concentration) in a total volume of 180 μL. Following a 1-h incubation at room temperature to reach equilibrium of the enzyme·inhibitor complex, 20 μL of 5 mM Spectrozyme fXa was added and the steady-state rate of product formation measured by monitoring the change in absorbance at 405 nm. The data were fit by nonlinear regression analysis to eq 1, and values for K_i^* were determined. The K_m for Spectrozyme fXa with FXa under these conditions was determined to be 67 μM by fitting the data to the Michaelis–Menton equation and is in good agreement with the value of 65 μM previously determined (Jordan et al., 1990).

Determination of Association Rate Constants. The association rate constants of ecotin with FXa were determined as follows. At reference time zero, ecotin (7 nM final concentration) was added to FXa (5 nM final concentration) and TNCT buffer in a total volume of 3 mL. Immediately thereafter, 150-μL aliquots were removed at intervals over a period of 10 min and added to microtiter wells containing 50 μL of 5 mM Spectrozyme fXa. The absorbance of the wells was monitored at 405 nm. Initial rates were determined for each well using the initial linear portion of each data set. The concentration of free enzyme versus time was plotted and the data fitted to eq 4; values for k_{on} were determined by nonlinear regression analysis.

Determination of Dissociation Rate Constants. The dissociation rate constant of the ecotin·FXa complex was measured using HLE to trap the free ecotin released from the complex. FXa (10 μM) was incubated for 1 h in TNCT buffer in the presence and absence of ecotin (12.5 μM) and diluted 1000-fold into TNCT buffer with and without HLE (500 nM). Aliquots (180 μL) were removed at various times, and initial rates were measured at 405 nm with 20 μL of 5 mM Spectrozyme fXa. The product curve data (free FXa versus time) was fit to eq 6; the value for k_{off} was determined by nonlinear regression analysis. A control lacking ecotin showed that the specific activity of FXa was unaffected in the presence of a 50-fold excess of HLE over the time course of the experiment.

Specificity Assays. The following assays were used to test the specificity of ecotin and the M84K and M84R mutants against various proteases; the inhibitors (100 nM each) or a buffer control were incubated at room temperature for ca. 1 h with each enzyme prior to substrate addition. The enzymes tested (enzyme concentration, buffer, substrate) were FXa (1.2 nM, buffer A, 0.7 mM Spectrozyme fXa), thrombin (3.7 nM, buffer A, 0.7 mM S2366), TF·FVIIa (12.0 nM, buffer B, 0.7 mM S2366), FXIa (1.2 nM, buffer A containing 1 mg/mL BSA, 0.7 mM S2366), activated protein C (4.3 nM, buffer A, 0.7 mM S2366), plasmin (15 nM, buffer A, 1 mM S2251), single chain t-PA (15 nM, buffer A, 2.5 mM S2288), factor XIIa (10 nM, buffer A, 0.5 mM S2302), plasma kallikrein (10 nM, buffer A, 0.5 mM S2302), HLE (17 nM, 0.1 M Tris, pH 7.5, 0.5 M NaCl and 0.005% Triton X-100, 0.42 mM MeOSuc-AAPV-pNA), bovine trypsin (10 nM, buffer A, 0.25 mM Spectrozyme fXa), and bovine chymotrypsin (10 nM, buffer A, 1 mM Suc-AAPF-pNA). Buffer A contained 50 mM Tris, pH 7.5, 100 mM NaCl, 2 mM $CaCl_2$, and 0.005% Triton X-100. Buffer B contained 50 mM Tris, pH 7.5, 100 mM NaCl, 10 mM $CaCl_2$, 0.5% BSA, 60 nM TF_{1-243}, and 1 mM CHAPS. The initial change in absorbance at 405 nm was monitored after addition of substrate. Controls lacking inhibitor and/or enzyme were also assayed to assess the uninhibited rates and the background substrate hydrolysis rates, respectively.

Coagulation Assays. Clotting time assays were performed using the ACL 300 Research Coagulation Analyzer. For the prothrombin time (PT) assays, the incubation time was set at 120 s and acquisition time at 120–600 s depending on the expected outcome of the assay. Membranes from 293 cells expressing TF (Paborsky et al., 1989) were premixed with $CaCl_2$. The sample (plasma and inhibitor) and reagent ($CaCl_2$/TF) were automatically mixed together after a 2-min

[2] The P_1 residue refers to the position preceding the scissile peptide bond of the substrate or inhibitor as defined by Schechter and Berger (1967).

incubation at 37 °C. The clotting time was determined by optical assessment. The total incubation time of inhibitor with plasma before addition of $CaCl_2$/TF was ca. 5 min. Final concentrations were 78 nM to 7.8 μM ecotin, 3.7 nM TF (0.9 μg/mL by protein content), 22.5 mM $CaCl_2$, and 50% plasma in a total volume of 160 μL.

For the activated partial thromboplastin time (APTT) assays, the activation time was set at 120 s and acquisition time at 300–600 s depending on the expected outcome of the assay. Citrated normal human plasma and inhibitor were incubated together. The sample (plasma and inhibitor) and activator (Instrumentation Laboratories Ellagic acid/Phospholipid mix Test Reagent) were automatically pipetted and incubated together for 2 min at 37 °C, and then $CaCl_2$ was added and clotting time determined by means of optical assessment. The total incubation time of inhibitor with plasma was ca. 3 min before addition of activator and 5 min before addition of $CaCl_2$. Final concentrations were 0.57 nM to 5.7 μM ecotin, 15.3 μg protein/mL 293 cell membranes, 8.3 μM ellagic acid, 8.3 mM $CaCl_2$, and 33.3% plasma in a total volume of 162 μL.

Cleavage of Ecotin by FXa and Active Site Determination. In order to determine if ecotin is cleaved by FXa, enzyme and inhibitor were incubated together at pH 4 and 7.5 essentially as described by McGrath et al. (1991b). At various time points, aliquots of the reaction mixture were removed and subjected to SDS–PAGE on 16% Tricine gels. These gels were then electroblotted onto polyvinylidene difluoride (PVDF) membranes. The membranes were stained for 30 s in 0.1% Coomassie Blue R-250 in 50% methanol, destained, and the bands of interest excised and sequenced.

Amino Acid Composition, Sequence Analysis, and Mass Spectrometry. Amino acid composition analysis and sequential Edman degradation, performed directly on the PVDF membrane, were carried out as previously described (Seymour et al., 1990). Electrospray mass spectra were obtained using a Sciex API-III triple quadropole mass spectrometer equipped with the Ionspray interface operating at 5 kV and calibrated with a solution of poly(propylene glycols).

Molecular Weight Determination of Ecotin·FXa Complex. Size exclusion chromatography was performed with two TSK G3000SW-XL columns linked in series at a flow rate of 0.5 mL/min in 50 mM Bis-Tris and 0.5 M NaCl, pH 6.5 and monitored at 214 and 280 nm. The molecular weight standards (M_r) used to calibrate the columns were sweet potato β-amylase (200 000), bovine γ globulin (158 000), bovine serum albumin (68 000), human FXa (46 000), chicken ovalbumin (44 000), ecotin dimer (32 200; see below), human γ interferon dimer (29 000), equine myoglobin (17 000), and cytochrome *c* (12 500). Standards were from Sigma, Boehringer Mannheim, and Bio-Rad; γ interferon was from Genentech. Ecotin was loaded at 140 μM. Ecotin and FXa (10 μM each) were incubated in TNCT buffer for 10 min at room temperature prior to injection. The molecular weight of the ecotin·FXa complex was calculated from linear regression analysis of a plot of the log molecular weight versus ratio of elution volume to void volume (V_e/V_o).

Determination of Ecotin Dimer Dissociation Constant by Fluorescence Titration. The change in fluorescence of ecotin with concentration was carried out on an SLM Model 8000 spectrofluorimeter with excitation at 280 nm and emission at 340 nm. Ecotin (10 μM) and L-tryptophan (40 μM) were each consecutively diluted 2-fold in 50 mM Tris, 150 mM NaCl, and 2 mM $CaCl_2$, pH 7.5. The absorbance of the initial tryptophan solution was equivalent to that of the initial ecotin solution (A_{280} = 0.25) in order to compensate for any inner filter effects at higher concentrations. Measurements were taken within 15 s of dilution. The concentration dependence of the ratio of the ecotin fluorescence to the L-tryptophan fluorescence was used to determine the dissociation constant for ecotin dimerization.

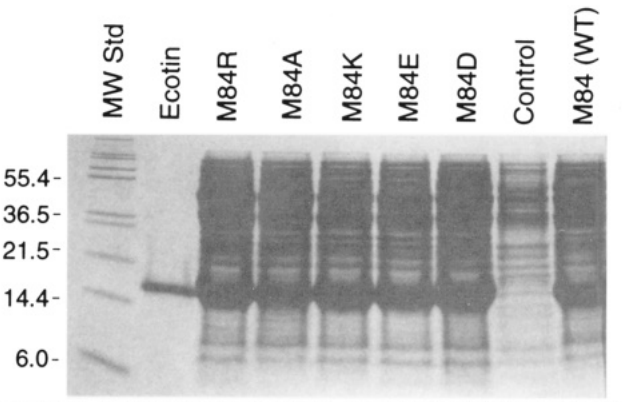

FIGURE 1: SDS–PAGE of purified ecotin and crude periplasmic proteins from *E. coli* transformed with ecotin plasmids. (Lane 1) Molecular weight markers (Novex Mark 12) with the associated M_r values × 10^{-3}. (Lane 2) Purified recombinant ecotin, 4 μg. (Lanes 3–7) 25 μL of periplasmic contents from ecotin mutant cultures; sample preparation is described in text. (Lane 8) 27C7 control (no plasmid). (Lane 9) 25 μL of periplasmic contents from wild-type ecotin culture. Samples were reduced with β-mercaptoethanol prior to loading, and the gel was stained with Coomassie Blue.

RESULTS

Cloning, Mutagenesis, and Expression of Ecotin. The ecotin gene was cloned by PCR using *E. coli* chromosomal DNA and synthetic primers based upon the published coding sequence (McGrath et al., 1991b; Lee et al., 1991). The resulting expression plasmid, pEt3, placed transcription of the ecotin gene under the control of the alkaline phosphatase promoter and translation under control of the tryptophan Shine–Dalgarno sequence. The endogenous ecotin signal sequence was used for efficient secretion of ecotin into the *E. coli* periplasm. Changing the ecotin P_1 residue Met 84 (see below) to Arg, Lys, Ala, Glu, or Asp resulted in mutants that were expressed in shake flasks at levels comparable to wild type; on the basis of SDS–PAGE, we estimate that ecotin represents ca. 15% of the total periplasmic protein (Figure 1). Upon comparison of the expression of recombinant ecotins to endogenous ecotin in the untransformed host, we estimate the endogenous level to be <1% that of the recombinant ecotins (Figure 1, lane 8). Recombinant wild-type ecotin was also expressed at the 10-L scale in order to isolate larger amounts of protein.

Purification of Ecotin. Approximately 650 mg of recombinant ecotin per 100 g of wet cell paste was purified from the periplasm of *E. coli* grown in a 10-L fermentor by chromatography on DEAE-Sepharose, reverse-phase C18 resin, Superdex size exclusion resin, and Q-Sepharose. Recombinant ecotin and ecotin mutants were also purified from shake flasks by chromatography on a trypsin affinity column followed by reverse-phase C18 HPLC, resulting in ca. 1 mg of isolated protein per liter of culture. Purification of the M84R and M84 K mutants yielded only ca. 0.1 mg of isolated protein per liter of culture, due to cleavage of ecotin at P_1 (Lys or Arg) by the immobilized trypsin; the cleaved protein was readily resolved from intact material by HPLC.

Purity, Mass Spectroscopy, and Amino Acid Analysis. The mass spectral data (16099.3 amu, obsd.; 16097.5 amu, calcd.

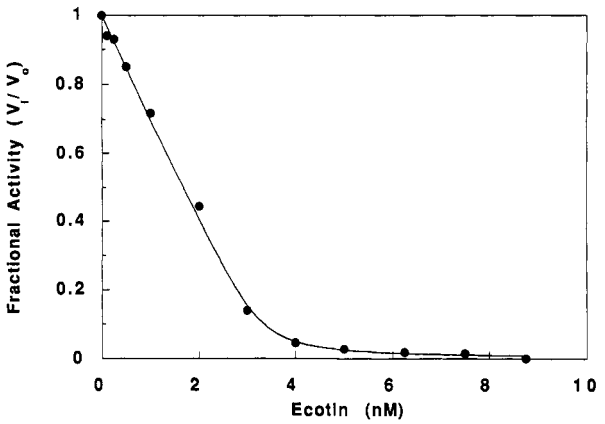

FIGURE 2: Determination of the apparent equilibrium dissociation constant of ecotin with FXa. The inhibitory activity is expressed as the ratio of the inhibited rate to the uninhibited rate (fractional activity) at varying inhibitor concentrations at equilibrium. The FXa concentration was 3.3 nM. The apparent equilibrium dissociation value was determined by nonlinear regression analysis of the data using eq 1 and yielded a K_i^* value of 47 pM. The line represents the fractional activity determined from eq 1 and the calculated K_i^*. The data shown are typical of three independent determinations.

for ecotin containing one disulfide bond) and amino acid analysis of purified recombinant ecotin was identical to that predicted from the DNA sequence. The purified ecotin was >98% homogeneous based on SDS–PAGE (Figure 1, lane 2) and reverse-phase HPLC. Mass spectral analysis of the purified M84R, M84K, M84A, M84D, and M84E mutants revealed masses within 2 amu of the respective calculated masses for each protein. Amino acid analysis of the mutants was within experimental error of that calculated from the sequence (data not shown).

Determination of Equilibrium Dissociation and Kinetic Constants. Since there was substantial FXa inhibition at concentrations of ecotin comparable to that of FXa, Michaelis–Menton kinetics are not valid. Therefore, apparent equilibrium dissociation values (K_i^*) were determined using methods derived for tight-binding inhibitors (Morrison, 1969; Bieth, 1974; Williams & Morrison, 1979). The inhibition of FXa by ecotin under equilibrium conditions is shown in Figure 2. The data were fit by nonlinear regression analysis to

$$V_i/V_o = 1 - \frac{[E_o] + [I_o] + K_i^* - \sqrt{([E_o] + [I_o] + K_i^*)^2 - (4[E_o][I_o])}}{2[E_o]} \quad (1)$$

where V_i/V_o is the fractional activity (steady-state inhibited rate divided by the uninhibited rate), $[E_o]$ is the total FXa concentration, and $[I_o]$ is the total ecotin concentration. In this manner, a K_i^* value of 54 pM was calculated for ecotin binding to FXa. The introduction of positively charged residues in the P_1 position led to mutants with slightly higher affinity for FXa, whereas negatively charged residues substantially decreased this affinity. Replacement of M84 with alanine resulted in a 230-fold reduction in binding affinity. The K_i^* values for the ecotin mutants are reported in Table 1.

If the inhibitor and substrate compete for the same active site, which is almost certainly true since ecotin is cleaved by the enzyme (see below), the true K_i value is generally related to K_i^* by the expression $K_i = K_i^*/(1 + [S]/K_m)$, where K_m is the Michaelis constant for Spectrozyme fXa (Bieth, 1980).

Table 1: Equilibrium Dissociation and Kinetic Constants for Ecotin and P_1 Mutants with Factor Xa

ecotin mutant	K_i^* (pM)[a]	$k_{on} \times 10^{-6}$ (M^{-1} s^{-1})[b]	$k_{off} \times 10^5$ (s^{-1})[c]
wild type	54 ± 13	1.35 ± 0.08	6.5 (7.3)
M84R	11	0.28 ± 0.01	(0.3)
M84K	21	0.68 ± 0.02	(1.4)
M84A	3900	0.17 ± 0.01	(66)
M84D	55 200	ND[d]	ND
M84E	25 600	0.07	(179)

[a] The wild-type K_i^* represents the average of three determinations; the mutant K_i^* values are from single determinations. [b] Represents the average of three determinations except for M84E, which was from a single determination. [c] Values in parentheses are calculated from $(K_i^*)(k_{on})$. [d] ND, not determined.

However, ecotin·FXa complex dissociation is negligible during the assay (see below). Furthermore, the K_i^* was independent of substrate concentration (data not shown), confirming that no substrate-induced dissociation occurred. In this instance, the K_i^* for ecotin and the P_1 Arg and Lys mutants is equal to K_i; for the P_1 Ala, Asp, and Glu mutants where dissociation is faster, the above correction may apply.

Determination of Association Rate Constants. The rate of association of a reversible inhibitor with an enzyme is given by

$$\frac{-d[E]}{d(t)} = k_{on}[E][I] - k_{off}[EI] \quad (2)$$

Since measurements were made during the initial phase of the reaction, where dissociation of the E·I complex is negligible, the second term of eq 2 can be ignored, and the rate of association reduces to

$$\frac{-d[E]}{d(t)} = k_{on}[E][I] \quad (3)$$

When $E_o \neq I_o$, this integrates to

$$[E] = [E_o] - \frac{[I_o][E_o](e^{\{([I_o]-[E_o])k_{on}t\}} - 1)}{[I_o]e^{\{([I_o]-[E_o])k_{on}t\}} - [E_o]} \quad (4)$$

where [E] is the concentration of free enzyme at any time t and $[E_o]$ and $[I_o]$ represent the initial concentrations of enzyme and inhibitor, respectively. The association rate constant for ecotin with FXa was determined by measuring free enzyme as a function of time and fitting the data to eq 4 by nonlinear regression analysis. A value for k_{on} equal to 1.35 ± 0.08 × 10^6 M^{-1} s^{-1} was calculated for ecotin with FXa (Figure 3A); k_{on} values for ecotin mutants, which were all somewhat less than wild type, are reported in Table 1.

Determination of Dissociation Rate Constants. To measure the dissociation rate constant of ecotin from FXa, the complex was diluted 1000-fold into buffer containing a 50-fold excess of HLE to trap the free ecotin and prevent reassociation with FXa. In the absence of HLE, reassociation of ecotin with FXa was so rapid that free FXa was barely detectable (Figure 3B). Progress of dissociation was monitored as the increase in hydrolysis rate of Spectrozyme fXa, which is not hydrolyzed by HLE, resulting from the increasing free FXa with time. The specific activity of FXa in the absence of ecotin was unaffected by HLE. Because HLE prevented reassociation, the rate of dissociation of the E·I complex is given by

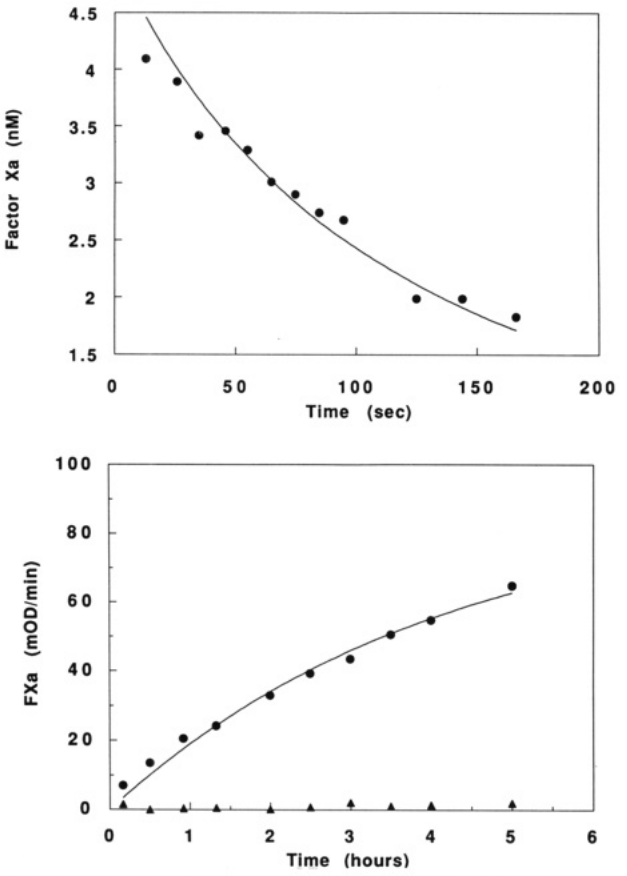

FIGURE 3: Determination of ecotin and FXa association and ecotin·FXa dissociation rates. Experimental conditions, curve fitting, and rate constant determinations are described in the text. (Panel A, top) Measurement of the association rate constant of ecotin with FXa. The curve results from nonlinear regression analysis of the data fit to equation 4. (Panel B, bottom) Measurement of the dissociation rate constant of ecotin from FXa in the presence of HLE (●) and in the absence of HLE (▲). The curves result from nonlinear regression analysis of the data fit to eq 6, where the measured velocity is proportional to free [FXa].

$$\frac{d[E]}{d(t)} = k_{off}[EI] \tag{5}$$

which upon integration gives

$$[E] = [EI]_0(1 - e^{-k_{off}t}) \tag{6}$$

where [E] is the free [FXa] at any time t, $[EI]_0$ is the concentration of the complex at time zero, and k_{off} is the first-order dissociation rate constant. The time-dependent ecotin·FXa complex dissociation is shown in Figure 3B; the dissociation rate constant determined from fitting the data to eq 6 by nonlinear regression analysis is 6.5×10^{-5} s^{-1} (Table 1).

Cleavage by Factor Xa and Reactive Site Determination. The interaction of ecotin with FXa results in a slow cleavage of the inhibitor by the enzyme under certain *in vitro* conditions. Figure 4 illustrates the amount of cleavage of ecotin by trypsin and by FXa at pH 4.0 in 139 h. The inhibitor is less sensitive to cleavage by trypsin than by FXa under these conditions. The same experiment conducted at pH 7.5 showed a similar relationship between trypsin and FXa cleavage, although the total amount of cleavage occurring at neutral pH was much less than that occurring at pH 4.0, where hydrolysis is favored (data not shown). Arrows indicate the bands which were later excised from the blot and sequenced. The N-terminal

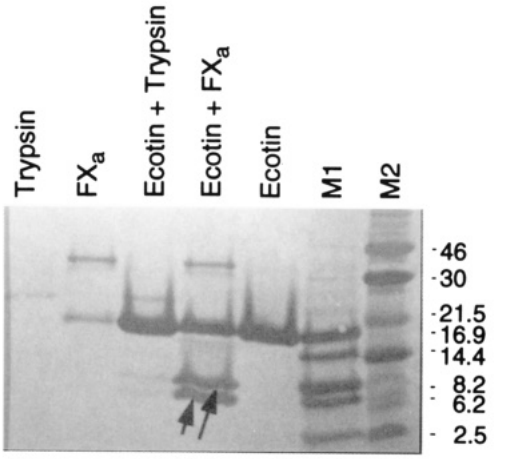

FIGURE 4: Cleavage of ecotin by FXa and trypsin and reactive site determination. Samples were incubated as described in the text for 139 h at room temperature, reduced, run on 16% Tricine gels and blotted onto PVDF. (Lane 1) Trypsin. (Lane 2) FXa. (Lanes 3 and 4) Ecotin with trypsin and FXa, respectively. (Lane 5) Ecotin. (Lanes 6 and 7) Horse myoglobin peptides (LKB) and Rainbow (Amersham) molecular weight markers, respectively, with the associated M_r values $\times 10^{-3}$. The amino-terminal sequence of the lower band in lane 4 (ca. 6.2 kDa) was MA-PDGKKEK; the amino-terminal sequence of the upper band in lane 4 (ca. 8.2 kDa) was AESVQ, corresponding to the amino terminus of ecotin.

Table 2: Percent Activity of Selected Proteases in the Presence of Ecotin and the M84R and M84K P_1 Mutants[a]

protease	ecotin	M84R	M84K
factor Xa	0	1	2
thrombin	98	4	16
TF·factor VIIa	99	87	92
factor XIa	79	5	12
activated protein C	99	50	65
plasmin	100	10	9
t-PA	100	100	100
factor XIIa	0	1	0
kallikrein	0	1	2
HLE	0	88	85
trypsin	0	0	0
chymotrypsin	0	0	1

[a] Conditions for the inhibition assays are described under Materials and Methods.

sequences obtained (Figure 4) revealed that FXa cleaves ecotin between M84 and M85.

Specificity. In order to investigate the activity and specificity of ecotin more completely, we assayed ecotin and the M84R and M84K P_1 mutants with several other human serine proteases found in plasma. In addition to FXa, ecotin potently inhibited FXIIa and kallikrein, as well as HLE (K_i values <1 nM, manuscript in preparation); little or no inhibition was observed with thrombin, TF·FVIIa, FXIa, activated protein C, plasmin, or t-PA (Table 2). Changing the P_1 residue to either Arg or Lys led to mutants that were slightly more potent as FXa inhibitors (Table 1). These mutants also exhibited significant inhibition of thrombin, FXIa, activated protein C, and plasmin. FXIIa and kallikrein were still potently inhibited; however, the introduction of the positively charged Arg or Lys in the P_1 position effectively diminished inhibition of HLE (Table 2). No inhibition by ecotin or either mutant was observed with TF·FVIIa or t-PA, whereas bovine trypsin and chymotrypsin were potently inhibited by all three inhibitors (Table 2).

Determination of Ecotin·FXa Complex Formation. Ecotin and FXa were incubated together at 10 μM each for 10 min to form an ecotin·FXa complex, which was characterized by

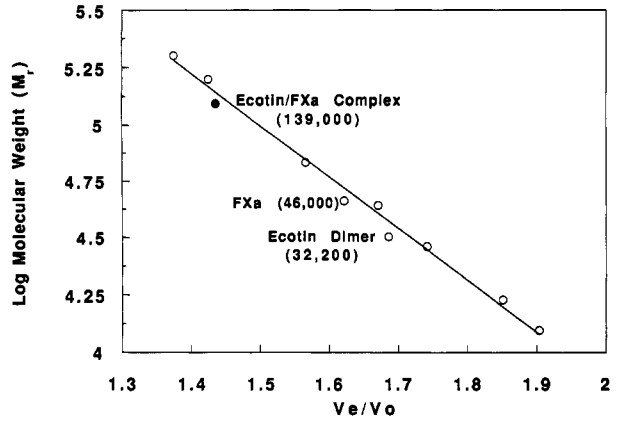

FIGURE 5: Determination of the molecular weight of the ecotin·FXa complex by gel filtration. The ratio of elution volume/void volume (V_e/V_o) is plotted versus log molecular weight for the ecotin·FXa complex (●) and molecular weight standards (○). The line represents a least-squares fit to the data. The ecotin·FXa complex is plotted according to the calculated molecular weight of 124 200 for the $(ecotin)_2$·$(FXa)_2$ complex. Selected molecular weights are noted in parentheses. Conditions for complex formation and chromatography are described under Materials and Methods.

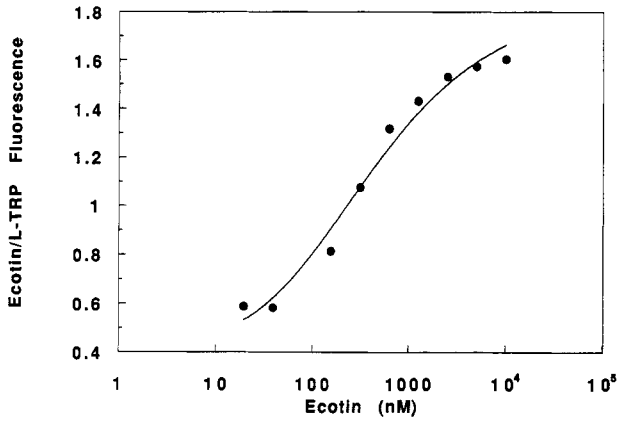

FIGURE 6: Fluorescence titration of ecotin. The ratio of ecotin fluorescence to that of tryptophan fluorescence is plotted versus ecotin concentration. The curve represents a nonlinear regression analysis of the data fit to eq 9.

gel filtration on HPLC TSK columns. The molecular weight (M_r) of the complex, determined from a plot of log molecular weight versus V_e/V_o (Figure 5), was 139 000. This is consistent with the formation of an $(ecotin)_2$·$(FXa)_2$ complex, which has a calculated M_r of 124 200. FXa eluted near its M_r of 46 000, whereas ecotin eluted as a dimer with a M_r of 32 200; ecotin monomer has a calculated M_r of 16 097.5.

Determination of Ecotin Dimerization Dissociation Constant. The dimerization of ecotin, which contains two tryptophans, was followed by measuring the change in fluorescence upon dilution compared to that of L-tryptophan. The rate of ecotin dimer dissociation was too fast to be measured. A sigmoidal dependence for the ratio of the ecotin/L-tryptophan fluorescence versus concentration was observed which is consistent with a two-state model (Figure 6). For the case of ecotin monomer (I) in equilibrium with dimer (I_2), $I + I \rightleftharpoons I_2$, where the equilibrium constant $K_d = [I]^2/[I_2]$. The total concentration of ecotin ($[I_T]$) is defined as $[I_T] = [I] + 2[I_2]$. Combining these two equations and dividing by I_T results in

$$\text{Fraction monomer} = [I]/[I_T] = \{-K_d + (K_d^2 + 8K_d[I_T])^{1/2}\}/4[I_T] \quad (7)$$

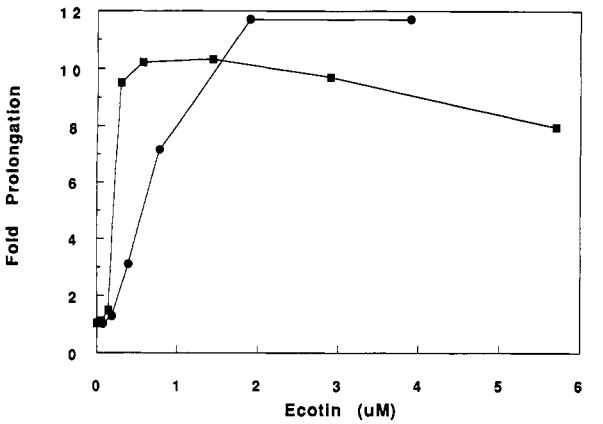

FIGURE 7: Prolongation of clotting time in normal human plasma. The fold prolongation in clotting time is shown upon initiation by TF in the PT assay (●) or ellagic acid in the APTT assay (■). The uninhibited clotting times were 48 and 31 s for the PT and APTT, respectively. Conditions for the assays are given under Materials and Methods.

In addition

$$[I]/[I_T] = 1 - (Fl_X - Fl_M)/(Fl_D - Fl_M) \quad (8)$$

where Fl_M and Fl_D are the relative fluorescence (ecotin fluorescence/L-tryptophan fluorescence) of ecotin monomer and dimer, respectively, and Fl_X is the observed relative fluorescence. Combining eqs 7 and 8 results in

$$Fl_X = Fl_M + (Fl_D - Fl_M)\{-K_d + (K_d^2 + 8K_d[I_T])^{1/2}\}/4[I_T] \quad (9)$$

A nonlinear fit of the data to eq 9 results in a calculated K_d value for ecotin dimerization of 390 ± 150 nM.

Prolongation of Clotting Time. Ecotin caused a 10-fold prolongation of clotting times at concentrations of ca. 0.3 μM and ca. 2 μM in APTT and PT assays, respectively (Figure 7). The dose–response curve for the the APTT assay was particularly steep, with clotting times increasing from 1.5-fold to ca. 10-fold prolongation over a range of only 0.2–0.3 μM. The dose–response curve for the PT assay was considerably less steep, requiring a concentration range of 0.2–2 μM for the same effect in prolongation of clotting time.

DISCUSSION

Ecotin is a potent anticoagulant and the most potent reversible inhibitor of FXa described to date with an equilibrium dissociation constant (K_i) of 54 pM. Due to the high affinity, equilibrium dissociation constants were determined using methods described for tight-binding inhibitors[3] (Morrison, 1969; Bieth, 1974; Williams & Morrison, 1979). The reversible inhibition of ecotin (I) with FXa (E) is shown in Scheme 1, a scheme from which eq 1 has been derived. This scheme represents the simplest possible mechanism consistent with the observed data; however, it is possible that more complex multistep mechanisms involving the formation of an E·I intermediate(s) may be involved as has been observed for many slow, tight-binding inhibitors (Laskowski & Kato, 1980; Morrison, 1982).

[3] Due to limitations of the signal to noise, the equilibrium dissociation measurements were determined at enzyme concentrations of 20–100-fold above the K_i. Although it is difficult to obtain accurate data at this enzyme concentration, the K_i of 54 pM measured under equilibrium conditions is in excellent agreement with the K_i calculated by k_{off}/k_{on} of 48 pM.

Scheme 1

$$E + I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} E{\bullet}I$$

The association of ecotin with FXa is rapid with an association rate constant (k_{on}) of 1.35×10^6 M^{-1} s^{-1}. Experiments designed to directly measure the rate of dissociation of the ecotin·FXa complex were unsuccessful due to the rapid reassociation of free ecotin with FXa, resulting in insufficient free FXa to be measured accurately (Figure 3B). Therefore the dissociation rate was measured in the presence of a 50-fold excess of HLE, which binds ecotin with high affinity (K_i < 1 nM, data not shown), thus preventing reassociation with FXa. HLE does not hydrolyze Spectrozyme fXa, nor does it affect the specific activity of FXa. Although we cannot rigorously exclude the possibility that HLE affects the dissociation of ecotin from FXa, this is unlikely since the k_{off} value calculated from $(K_i)(k_{on})$ is in excellent agreement with that measured kinetically in the presence of HLE (Table 1). Since the ecotin·FXa complex for the dissociation rate measurements was formed at concentrations where ecotin is a dimer (i.e., $E_2{\cdot}I_2$; see below), the mechanism of dissociation could involve a slow step of ecotin dissociation to form the E·I complex, followed by a fast step of FXa dissociation from ecotin. However, this is unlikely since the rate of dissociation of the ecotin dimer itself is rapid relative to the rate of dissociation of the ecotin·FXa complex.

On the basis of active site titration, the inhibition of FXa by ecotin at high concentrations (>100K_i) indicated that the EI complex formed was stoichiometric, i.e., a 1:1 molar ratio of FXa to ecotin was found. The methods used to calculate the equilibrium and kinetic constants assumed that this complex contained one molecule each of ecotin and FXa (Scheme 1). However, the interaction of ecotin with FXa at high concentrations (10 μM range) was shown by gel filtration chromatography to elute with a molecular weight corresponding to a tetrameric complex consisting of two molecules of ecotin and two molecules of FXa (Figure 5). Ecotin has previously been observed to form a complex with trypsin with similar stoichiometry (Chung et al., 1983). A mechanism for this complex formation is presented in Scheme 2.

Scheme 2

$$\begin{array}{ccccc} & & I \rightleftharpoons E{\bullet}I & & E{\bullet}I \\ & + & & \searrow & \\ E & \downarrow\uparrow I & & \downarrow\uparrow I & E_2{\bullet}I_2 \\ & + & & \swarrow & \\ & & I_2 \rightleftharpoons E{\bullet}I_2 & & E \end{array}$$

Scheme 2 is supported by gel filtration chromatography data (Figure 5) which show that ecotin forms a dimer at a concentration of 140 μM; no evidence for dimer formation of FXa alone was observed. The tetrameric complex that we observed at high concentrations likely results from the sequential addition of two FXa molecules to the ecotin dimer. This scheme is further supported based on evidence from ecotin fluorescence data (Figure 6). Ecotin contains two tryptophans, at least one of which undergoes a fluorescence intensity change upon dilution. Fitting the data to a two-state monomer/dimer model ($2I \rightleftharpoons I_2$) results in a K_d value for ecotin dimerization of ca. 390 nM. This value supports the observation that two molecules of FXa bind to the ecotin dimer since the gel filtration experiments were carried out at concentrations above the K_d for ecotin dimerization (Figure 5). Furthermore, since equilibrium and kinetic measurements were conducted at a concentration range well below the K_d for ecotin dimerization, the assumptions used in Scheme 1 and eq 1 to calculate these values are valid.

The interaction of ecotin with FXa at pH 4 results in a slow cleavage of the inhibitor between M84 and M85.[4] This occurs more rapidly with FXa than with trypsin (Figure 4); very little cleavage was observed with either enzyme at pH 7.5. These results concur with earlier data which demonstrated that M84 is the P_1 reactive site for ecotin with trypsin, chymotrypsin, and porcine pancreatic elastase (McGrath et al., 1991b). Factor Xa is a trypsin-like serine protease whose substrates, including prothrombin and chromogenic peptides (Lottenberg et al., 1981), and whose inhibitors, including ATIII (Björk & Danielsson, 1986), TFPI (Broze et al., 1990), antistasin (Dunwiddie et al., 1989), and most likely TAP (Dunwiddie et al., 1992), possess an Arg in the P_1 position. We therefore made and tested a number of mutations at the ecotin P_1 residue to see what effect they would have on FXa binding affinity as well as any specificity changes toward other serine proteases. Of the five ecotin mutants which were constructed, two (M84R and M84K) were found to be slightly better FXa inhibitors than wild type (Table 1). This increase in affinity is due to slower dissociation rates since the association rate of wild type with FXa is slightly faster than those of the mutants (Table 1).

Surprisingly, even when the P_1 residue was changed to Asp or Glu, significant inhibition of FXa activity was observed, suggesting that residues other than P_1 make important contributions to binding. It is unlikely that the observed inhibition of FXa with these mutants is due to contaminating endogenous ecotin. Based upon calculations using eq 1 and solving for the concentration of inhibitor required to obtain the fractional activity observed with either M84D or M84E, the amount of endogenous ecotin would have to be ca. 10% of the total. However, the expression of these mutants is at least 100-fold above the endogenous level of ecotin expression (Figure 1). Furthermore, no evidence for wild-type ecotin was seen in the mass spectral data for purified M84D (16 081.5 amu); the mass of the M84E mutant is only 2 amu less than wild type and could not be distinguished by this criteria.

The substitution of either Arg or Lys for the P_1 Met residue resulted in an overall decrease in specificity as evidenced by increased inhibition of thrombin, factor XIa, plasmin, and, to a lesser extent, activated protein C (Table 2). As expected, the M84R and M84K mutants no longer inhibited HLE (Table 2). Thus, the net effect was to increase specificity for FXa over HLE, but decrease specificity for FXa relative to most of the plasma proteases tested. A similar phenomenon has been observed with α1-proteinase inhibitor, a serpin containing a Met in the P_1 position, which potently inhibits trypsin, HLE, and chymotrypsin. α1-Proteinase inhibitor (Pittsburgh), a naturally occurring mutant in which Arg replaces Met in the P_1 position, displays 10 000-fold decreased inhibition toward HLE but is a more potent inhibitor of trypsin and the plasma proteases thrombin, plasmin, FXa, factor XIa, kallikrein, factor XIIa, urokinase, and activated protein C (Travis et al., 1986; Scott et al., 1986; Patson et al., 1990; Heeb et al., 1990). In addition, both the Kunitz inhibitor BPTI and the Kazal inhibitor from turkey ovomucoid third domain show potent

[4] Although the FXa used was pure based on SDS–PAGE (data not shown), we cannot rigorously exclude the possibility that a contaminating protease was responsible for this cleavage.

inhibition of HLE when P_1 is Met which is greatly diminished when P_1 is Lys; the converse is found for trypsin (Beckmann et al., 1989; Bigler et al., 1993). No inhibition was observed with ecotin or the P_1 mutants and TF·FVIIa or single chain t-PA, suggesting that the active sites of these proteases differ significantly from the others or that a region distal from the active site on the proteases prevents ecotin binding.

Thrombosis is a complex process involving the coagulation and fibrinolysis systems, platelet adhesion and aggregation, and the endothelium (Badimon et al., 1991). Thrombin plays a central role in this process since it cleaves fibrinogen to fibrin, activates platelets, and interacts with the vessel wall. The regulation of thrombosis using thrombin inhibitors has been extensively studied; however, an alternative strategy is the inhibition of FXa to prevent thrombin formation. The anticoagulant potential of both synthetic and recombinant inhibitors of FXa and thrombin have been evaluated in various clotting assays (Hauptmann & Kaiser, 1993). Recent evidence has shown that FXa inhibitors increase t-PA induced thrombolysis and prevent reocclusion in a canine model of arterial thrombosis (Mellott et al., 1993) and also prevent venous thrombosis in a rabbit model (Vlasuk et al., 1991). Furthermore, FXa has been implicated in determining the procoagulant activity of whole-blood clots (Eisenberg et al., 1993). Therefore, the inhibition of FXa by agents such as ecotin represents an attractive approach for clinical intervention in various thrombotic disorders.

At functionally useful concentrations based on *in vitro* clotting assays (ca. 500 nM), the interaction of ecotin with FXa is essentially irreversible since this concentration is so much larger than the K_i (Bieth, 1984). Based on the kinetic parameters of ecotin with FXa and a maximum possible FXa concentration in blood of ca. 200 nM, the time for complete inhibition of FXa is approximately 7.4 s (Bieth, 1984). In addition, the ecotin·FXa complex is relatively stable *in vitro* with a half-life of ca. 3 h. Obviously, many biological and physiological factors can affect this inhibiton; however, the high affinity, fast on rate, and slow off rate predict that ecotin could play a significant role *in vivo*.

Although no equilibrium dissociation constants were determined, ecotin was previously found to inhibit trypsin, chymotrypsin, porcine pancreatic elastase, and rat mast cell chymase; no inhibition was observed with thrombin, tryptase, kallikrein,[5] plasmin, or a variety of microbial proteases including those found in *E. coli* (Chung et al., 1983). In our studies, we have observed potent inhibition by ecotin of plasma kallikrein as well as factor XIIa (manuscript in preparation). The inhibition of the contact activation proteases plasma kallikrein and FXIIa may in part explain the potent anticoagulant effect observed in the APTT assay (Figure 7), which is a measure of intrinsic coagulation pathway activation. Surface activation of FXII to FXIIa leads to the formation of FXIa and kallikrein. FXIa activates FIX to FIXa, which in the presence of FVIII leads to the formation of FXa and ultimately a fibrin clot. Kallikrein can further activate FXII to produce more FXIIa (Schmaier et al., 1987). Therefore a molecule showing coordinate inhibition of FXa, FXIIa, and kallikrein may be a more potent anticoagulant than one that only inhibits FXa. The prolongation in clotting time by ecotin in the PT assay, initiated by tissue factor (the extrinsic pathway), reflects inhibition of FXa since ecotin does not inhibit TF·FVIIa or thrombin. Furthermore, kallikrein also activates plasminogen to plasmin, catalyzes the release of the potent vasodilator bradykinin from high molecular weight kininogen, has been implicated in neutrophil activation, and may regulate complement activation (Schmaier et al., 1987). Therefore ecotin could play an important role in the regulation of inflammation and fibrinolysis as well as coagulation. Major clinical manifestations of contact activation include sepsis, disseminated intravascular coagulation, and adult respiratory distress syndrome (Bone, 1992).

The physiological role of ecotin remains unknown. However, since ecotin does not inhibit any known *E. coli* proteases, its location in the periplasm may indicate a role in protecting the cell against external proteases (Chung et al., 1983). The inhibition of the pancreatic enzymes which are present in the mammalian gastrointestinal tract supports this hypothesis. The potent inhibition by ecotin of FXa as well as plasma kallikrein and FXIIa was unexpected and is somewhat more difficult to rationalize. Since ecotin is not homologous to any other known protease inhibitors, the X-ray crystal structure of a protease–inhibitor complex (McGrath et al., 1994) may provide a greater understanding of the nature of the ecotin active site(s) and the mechanism of inhibition for FXa and other serine proteases.

[5] The source and type of kallikrein used in these experiments were not reported (Chung et al., 1983).

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