Site-directed mutagenesis of the leech-derived Factor Xa inhibitor antistasin

Probing of the reactive site

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Antistasin (ATS) is a leech-derived 119-amino-acid protein which exhibits potent and highly selective inhibition of coagulation Factor Xa. It inhibits Factor Xa according to a common mechanism of serine-proteinase inhibitors in which a conformationally rigid substrate-like reactive site is presented to the enzyme. In this study a recombinant version of ATS was expressed and purified utilizing a yeast expression system in order to probe the reactive site P₁ (Arg-34) and P₁' (Val-35) residues by site-directed mutagenesis. The results demonstrate the requirement for a positively charged residue in the P₁ position of ATS, with an arginine residue preferred over a lysine, yielding K_i values of 61 pM and 1.28 nM respectively. Mutation of the P₁ arginine residue to the non-polar amino acid leucine abolished its inhibitory potency toward Factor Xa. The role of the C-terminal domain of ATS, which shares significant amino acid sequence identity with the N-terminal domain, was investigated by creating a second reactive site in the corresponding position of the C-terminal domain. The inhibitory activity of this mutant demonstrated that the C-terminal domain of ATS is not folded into the proper conformation necessary to create a functional inhibitory domain.

INTRODUCTION

Blood coagulation involves a cascade of enzyme-catalysed reactions which ultimately lead to the formation of a stable fibrin clot. The activated serine proteinase Factor Xa (FXa) plays a pivotal role in the coagulation cascade, since it is positioned at the point where the intrinsic and extrinsic activation pathways converge. After activation, FXa associates with Factor Va and Ca²⁺ on an acceptable phospholipid surface to yield the prothrombinase complex, which directly catalyses the formation of thrombin from prothrombin. Thrombin subsequently converts fibrinogen into fibrin, which polymerizes to form a stable clot. The unique position of FXa in the coagulation cascade has led to the discovery of potent and specific polypeptide inhibitors of this serine proteinase [1–6].

One such inhibitor, antistasin (ATS), was isolated from the salivary glands of the Mexican leech *Haementeria officinalis* [1,2]. ATS is a 119-amino-acid cysteine-rich protein which is present as multiple isoforms in leech salivary extracts [2,7,8]. Although it displays no sequence similarity to any known protein, a striking feature of the ATS primary structure is a two-fold internal sequence similarity which suggests that the molecule is organized into two separate and distinct domains [2,7]. By utilizing a single recombinant isoform of ATS expressed in an insect baculovirus host-vector system (irATS), ATS has been shown to inhibit FXa according to a common mechanistic pathway used by most small protein inhibitors of serine proteinases [8,9]. This class of inhibitors binds very tightly to their target enzyme in a manner analogous to a good substrate, but are only slowly hydrolysed by partial proteolytic cleavage [10–12]. The peptide bond which is cleaved is referred to as the reactive site, with the P₁ residue generally reflecting the specificity of the cognate enzyme. The P₁ residue in ATS has been identified as Arg-34 [3,9], which corresponds to the specificity of the preferred substrates for FXa. Since the reactive site of ATS has been

localized to the N-terminal domain, the function of the corresponding C-terminal domain remains uncertain. To further our understanding of the reactive-site residues required for the inhibition of FXa, as well as probe the role of the C-terminal domain, the irATS cDNA was introduced into a yeast expression vector so that site-directed mutagenesis could be used to create single substitutions at desired positions within the molecule. We report here the results of these studies, which define those amino acids acceptable in the P₁ and P₁' positions of the reactive site for potent inhibition of FXa and discuss the resulting implications with respect to the possible function of the C-terminal domain.

MATERIALS AND METHODS

Construction of the ATS expression vector

The plasmid pKH4-3B [13] is a galactose-regulated high-copy-number yeast expression vector containing the phage f1 origin of replication which allows the production of single-stranded plasmid to facilitate site-directed mutagenesis. The pKH4-3B vector was digested with *Sph*I and the 3' extensions were made flush-ended with T₄ DNA polymerase. After digestion with *Bam*HI, the 13.3 kbp blunt-*Bam*HI pKH4-3B vector was agarose-gel-purified. The ATS coding sequence was isolated from a previously constructed expression vector [14] as a 344 bp *Xma*I-*Bam*HI fragment. An oligomer linker was synthesized with the sequence:

5'-CAAGGACCATTGGAC-3'

3'-GTTCTGTGTAACCTGGGCC-5'

This linker extends the ATS gene upstream from the *Xma*I site to the first codon of the mature ATS molecule, Gln¹. The 344 bp *Xma*I-*Bam*HI ATS fragment, the blunt-*Xma*I oligomer linker and the 13.3 kbp blunt-*Bam*HI pKH4-3B vector were combined in a three-way ligation to yield the plasmid pKH4-3B/ATS (Fig. 1 below). The sequence of the final construct was confirmed by sequence analysis.

Abbreviations used: ATS, antistasin; FXa, Factor Xa; irATS, single recombinant isoform of antistasin expressed in an insect baculovirus host-vector system; yrATS, yeast recombinant ATS; APTT, activated partial thromboplastin time.

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Construction of the antistasin mutants

The *Escherichia coli* strain CJ236 [*dut*, *ung*, *thi*, *relA*, *pCJ105* (*Cm^r*)] was transformed with the pKH4-3B/ATS plasmid. To facilitate site-directed mutagenesis, the pKH4-3B/ATS DNA was converted into the single-stranded form by infecting CJ236 transformants with M13K07 helper phage (Pharmacia) according to the manufacturer's directions. Precipitation of the phage, preparation of the single-stranded template DNA and the mutagenesis procedure were as described using the Muta-Gene M13 *in vitro* Mutagenesis Kit (Bio-Rad).

Six mutant versions of ATS were generated, using the following oligomer primers for mutagenesis (the amino acid changes are indicated by underbars):

1. 5'-GACAGTGCACCTTACATCTGACC-3' Arg³⁴ to Lys³⁴
2. 5'-GACAGTGCACGTTACATCTGACC-3' Arg³⁴ to Asn³⁴
3. 5'-GACAGTGCACCAAACATCTGACC-3' Arg³⁴ to Leu³⁴
4. 5'-GCGGACAGTGGATACGACATCTG-3' Val³⁵ to Ile³⁵
5. 5'-TTGGACAGGTAACTCTGCAGTTG-3' Lys⁹⁰ to Val⁹⁰
6. 5'-TTGGACAGGTGATTCTGCAGTTG-3' Lys⁹⁰ to Ile⁹⁰

The mismatches with the wild-type ATS sequence are underlined. All mutations were verified by the chain-termination DNA sequencing method using Sequenase (U.S. Biochemical Corp.).

Expression of wild-type and mutant ATS in yeast

The *Saccharomyces cerevisiae* strain JRY188 (*MAT α* , *sir3-8*, *leu2-112*, *trp1*, *ura3-52*, *his4*, *cir⁺*), kindly provided by Dr. J. Rine (University of California, Berkeley, CA, U.S.A.), was made *cir^o* by standard techniques. The JRY188 cells were transformed with the wild-type ATS vector pKH4-3B/ATS, and the six mutant ATS vectors according to the procedure of Hinnen *et al.* [15]. Transformants were selected on minimal-medium plates without leucine, and clonal isolates were propagated at 23 °C in 3X YEH medium [16] containing 4.8% (w/v) glucose for 24 h, followed by addition of 4.8% (w/v) galactose to the cell cultures for an additional 95–120 h. Under these conditions the GAL10 promoter is induced by galactose after the depletion of glucose from the growth medium. The resulting broth containing ATS was separated from the cells by centrifugation.

Purification of secreted yeast rATS (yrATS)

A litre portion of clarified culture medium containing yrATS was adjusted to a pH of 6.8 with NaOH and fractionated on an S-Sepharose Fast Flow (Pharmacia) cation-exchange column as described previously for the purification of irATS [8]. The peak FXa inhibitory fractions were pooled, concentrated in a Speed-Vac centrifuge and loaded directly on to a 0.46 cm × 15 cm Vydac C₄ column equilibrated with aq. 10% (v/v) propan-2-ol/0.1% trifluoroacetic acid at a flow rate of 1 ml/min. The column was developed with a linear gradient from aq. 10 to 25% propan-2-ol/0.1% trifluoroacetic acid over 25 min and the eluant monitored at 210 nm. The yrATS peaks were collected by hand, freeze-dried, resuspended in h.p.l.c.-grade water and stored at 4 °C.

Analytical evaluation of purified yrATS samples

yrATS concentrations and compositions were determined by quantitative amino acid analysis of 20 h-acid-hydrolysed samples using a Beckman model 6300 analyser. Direct N-terminal sequence analysis was resolved on an Applied Biosystems model 470A Sequenator. Analytical reverse-phase h.p.l.c. analysis was accomplished by using a 0.46 cm × 15 cm Vydac C₁₈ column and a Perkin–Elmer series 4 liquid chromatograph. The samples were

loaded in aq. 10% (v/v) acetonitrile/0.1% trifluoroacetic acid at a flow rate of 1 ml/min and eluted with a linear gradient from aq. 10 to 50% acetonitrile/0.1% trifluoroacetic acid over 40 min, and the eluant was monitored at 220 nm. Electrophoretic analysis of proteins was done by the method of Schagger & Von-Jagow [17].

Standard plasma-based clotting assay

The activated partial thromboplastin time (APTT) was measured on an MLA Electra 700 automated clot timer using the commercially available actin-activated cephaloplastin reagent (Dade, Aquada, Puerto Rico) and pooled normal human plasma (George King Biomedical, Overland Park, KS, U.S.A.). Serial dilutions of each yrATS sample were made in TBSA buffer [0.05 M-Tris/0.15 M-NaCl/0.1% (w/v) BSA, pH 7.5] and diluted 1:10 in freshly thawed plasma just before use. Clotting times were measured in s (maximum time of 150 s) in the absence of yrATS and at final yrATS concentrations ranging from 10 nM to 10 μ M.

Enzyme assays and measurement of the kinetic constants

Human FXa was prepared from human FX (Enzyme Research Laboratories, South Bend, IN, U.S.A.) as described previously [9,18], and the functional enzyme concentration was quantified by active-site titration using fluorescein mono-*p*-guanidino-benzoate [18]. The enzymic activity of human FXa was determined at 22 °C using the *p*-nitroanilide substrate Spectrozyme FXa (American Diagnostics, Greenwich, CT, U.S.A.) prepared as a stock solution in water. Reaction rates were measured in a 96-well microtitre plate (Dynatech, Chantilly, VA, U.S.A.) using a V-max kinetic microplate reader (Molecular Devices, Palo Alto, CA, U.S.A.) by monitoring the increase in *A*₄₀₅. Unless otherwise indicated, all reactions were performed in a final volume of 220 μ l of TBSA buffer with a final concentration of 500 pM-FXa and 300 μ M-substrate ($4 \times K_m$). Inhibited steady-state velocities were generated by preincubating FXa with increasing concentrations of yrATS (0.1–10 nM) for 40 min to allow equilibration of the enzyme–inhibitor (EI) complex. The reaction was initiated by the addition of substrate, and the residual enzyme activity was determined by measuring the initial velocity of the reaction over a 5 min period. The equilibrium dissociation constants were calculated by fitting the steady-state velocities to Morrison's equation for tight-binding inhibition using non-linear regression analysis [3,19]:

$$V_s/V_o = \{(E_t - I_t - K_i) + [(I_t + K_i - E_t)^2 + 4K_iE_t]^{1/2}\} / 2E_t$$

where K_i is the dissociation constant for the EI complex, V_s is the inhibited steady-state velocity, V_o is the control velocity (no inhibitor), I_t is the total inhibitor concentration and E_t is the total FXa concentration. Reiterative fits of the steady-state velocity data yielded independent measurements of K_i values with less than 15% error. Dissociation constants were determined in quadruplicate and expressed as the mean $\pm$ S.E.M.

The dissociation rate constant (k_{off}) was measured directly by preincubating yrATS and FXa at a 2:1 molar ratio for 40 min to allow equilibration of the EI complex. This mixture was then diluted 50-fold into assay buffer containing 300 μ M-substrate, and the rate of return of enzymic activity was measured. The absorbance tracings were fitted directly by non-linear regression to the integrated first-order rate equation described by Williams & Morrison [20,21] and Cha [22,23];

$$P = V_s t + (V_o - V_s)(1 - e^{-k_{\text{obs}} t}) / k_{\text{obs}}$$

where P is the measured absorbance resulting from substrate hydrolysis, V_o is the initial reaction velocity, V_s is the final steady-state velocity and k_{obs} is the apparent first-order rate constant

which describes the equilibration from the initial to the final steady-state, and in this case is equal to k_{off} . Dissociation rate constants were determined in quadruplicate and expressed as means $\pm$ S.E.M.

The kinetic parameters for two of the yrATS mutants, R34K and V35I, could not be accurately determined by measuring the steady-state velocities from pre-equilibrated EI complexes. The dissociation rates of the EI complex for these two mutant proteins were so high that dissociation of the EI complex over the time course of the assay became significant. Therefore, the kinetic constants for these mutant yrATS proteins were determined by generating slow-binding progress curves in which reactions were initiated by the addition of enzyme to mixtures of substrate and inhibitor [3,6]. The progress-curve data were fitted directly by non-linear regression to the integrated first-order rate equation described above to yield values of $k_{obs.}$ over a wide range of inhibitor concentrations. We have shown previously that ATS inhibits FXa according to a two-step mechanism described in Scheme 1 below, in which an initial EI complex is formed which slowly rearranges to form the more stable inhibited complex, EI* [3,24]:

Scheme 1

Scheme 1 can be described by the equation which relates the $k_{obs.}$ values determined above to the apparent equilibrium dissociation constant for the initial EI complex [21,24]:

$$k_{obs.} = k_2[I]/([I] + K_{i,app.}) + k_{-2}$$

where

$$K_{i,app.} = K_i(1 + [S]/K_m)$$

and

$$K_i = k_{-1}/k_1$$

The equilibrium constant defining the final EI complex (EI*) can be calculated from the expression:

$$K_{i,final} = K_i[k_{-2}/(k_2 + k_{-2})]$$

and the second-order rate constant (k_{on}) describing formation of EI* can be calculated from the relationship:

$$k_{on} = k_2/K_i$$

The dissociation rate constant, k_{off} , is equal to k_{-2} . The kinetic constants $K_{i,final}$, k_{off} and k_{on} were determined in triplicate and expressed as means $\pm$ S.E.M.

RESULTS AND DISCUSSION

Characterization of purified yrATS

To further our understanding of the residues in the ATS molecule responsible for the potent inhibition of FXa, a site-directed mutagenesis approach was desired. We have previously described the production and biochemical characterization of a single ATS isoform produced using an insect baculovirus expression system [8]. Although useful for the production of large quantities of recombinant proteins, the baculovirus expression system is a rather cumbersome method for the generation of

multiple mutant recombinant proteins. Therefore, the cDNA for a single ATS isoform was introduced into a yeast expression vector so that site-directed mutagenesis could be used to make single substitutions at desired positions within the molecule. The plasmid vector used to express and secrete yrATS, pKH4-3B/ATS, contains the GAL10 promoter, which allows expression induced by the addition of galactose (Fig. 1). This expression vector also includes the MF α 1 pre-pro secretory leader sequence with the KEX2 proteinase cleavage site (K-R) immediately upstream of the ATS sequence to allow for efficient secretion and processing of mature yrATS.

Titration of the FXa inhibitory activity in the culture medium revealed that yrATS expression had peaked at a level of approx. 1 mg/l after 120 h of fermentation. The recombinant protein was purified from 1 litre of fermentation broth utilizing S-Sepharose cation-exchange chromatography followed by C₄ reversed-phase h.p.l.c. essentially as described previously [8]. The h.p.l.c. purification step resolved two peaks of FXa-inhibitory activity at about a 1:1 ratio (peaks I and II), both of which exhibited the predicted amino acid composition. Analytical reversed-phase h.p.l.c. analysis demonstrated that peak II was eluted with the same retention time as the baculovirus produced irATS, whereas peak I had a slightly shorter retention time (Fig. 2). Direct N-terminal sequence analysis revealed that peak II, like irATS and native leech-derived ATS, was blocked at the N-terminus by a pyroglutamic acid residue. In contrast, peak I contained an unblocked N-terminal glutamine residue, the first amino acid predicted from the cDNA sequence. This variation in the N-terminal residue almost certainly explains the differing elution patterns obtained for yrATS peaks I and II on reversed-phase h.p.l.c., because the proteins are identical by all other analytical

Fig. 1. Plasmid vector used for the mutagenesis and expression of rATS in yeast

The coding sequence for the mature form of ATS was directionally cloned into the unique *Sph*I and *Bam*HI sites located between the MF α 1 pre-pro secretory leader (MF α 1sl) and the transcription termination sequences (MF α 1t). The GAL10 promoter-driven vector contains the entire yeast 2- μ DNA, the *leu2-d* gene for selection of LEU⁺ prototrophs and pBR322 sequences from the yeast shuttle vector pC1/1 [32]. The *E. coli*, *Saccharomyces cerevisiae* and f1 origins of replication (E ori, Y ori and f1 ori respectively) and the ampicillin-resistance marker (*Amp*^R) are indicated. The arrow denotes the direction of transcription.

Fig. 2. Analytical reversed-phase h.p.l.c. analysis of purified yrATS

A 10 μ g sample of irATS (a) and 20 μ g of the final preparation of h.p.l.c.-purified wild-type yrATS (b) were rechromatographed on an analytical C_{18} column. Peak I consists of the unblocked species and peak II represents the *N*-terminally blocked species of yrATS.

Table 1. Amino acid composition of purified yrATS peaks I and II

Amino acid	Composition†		
	Theoretical no. of residues*	Residues/molecule	
		Peak I	Peak II
Ala	3	3.1	2.9
Arg	12	10.8	11.0
Asx	9	8.9	9.2
Cys	20	ND†	ND
Glx	12	12.0	12.4
Gly	10	10.0	10.0
His	2	1.8	1.6
Ile	6	4.8	4.9
Leu	6	4.9	5.0
Lys	9	9.2	9.4
Met	3	2.5	2.5
Phe	3	2.9	2.7
Pro	9	11.0	10.0
Ser	6	5.0	5.0
Thr	5	5.2	5.4
Trp	0	ND	ND
Tyr	2	1.8	1.9
Val	2	2.0	2.0

* Number of each amino acid expected based on the cloned cDNA sequence.

† Normalized to the molar content of glycine (average of three independent analyses).

‡ ND, Residues destroyed during acid hydrolysis and thus were not determined quantitatively.

criteria, including amino acid composition analysis (Table 1), C-terminal sequence analysis and SDS/PAGE analysis (Fig. 3a). Since the levels of expression were so low in this system and the total protein produced was divided between two apparent species, we wanted to pool peaks I and II for all subsequent kinetic analysis. Before doing this we needed to determine whether both peaks were equipotent inhibitors of FXa. Each protein peak

Fig. 3. SDS/PAGE analysis of yrATS mutant preparations

(a) An 8 μ g portion of purified wild-type yrATS peak I or peak II was incubated with FXa for 24 h at 37 °C at a 10:1 molar excess of yrATS. The resulting cleavage products were analysed on 10–20% polyacrylamide/SDS/Tricine gels under reducing conditions and stained with Coomassie Blue. + and – indicates those samples incubated in the presence or absence of FXa respectively. Control lanes include irATS alone and FXa alone. M_r markers were run in parallel, and their associated M_r values are indicated. (b) A 5 μ g portion of each final purified mutant preparation containing a mixture of peaks I and II were fractionated as above and revealed by silver staining. Lane M contains the M_r markers.

from the wild-type yrATS preparation was purified to homogeneity by subsequent reversed-phase h.p.l.c. fractionation on a C_{18} column and analysed separately in an amidolytic FXa assay and a standard APTT clotting assay. Both yrATS peaks I and II yielded indistinguishable K_i values with FXa (51 pM and 55 pM respectively) and clotting-time profiles which overlapped one another (results not shown). Furthermore, when the recombinant proteins were incubated with catalytic quantities of FXa, they were cleaved to yield a single cleavage product with an equilibrium between cleaved and native inhibitor which was identical between peaks I and II (Fig. 3a). The sum of these results demonstrated that the *N*-terminally blocked and unblocked species of yrATS were equivalent with respect to their inhibitory

Fig. 4. Primary amino acid sequence of ATS and two-fold identity

The primary sequence of the ATS isoform used in these studies is shown with the two-fold internal sequence identity illustrated. Identical amino acid residues are boxed and two gaps introduced for maximum identity are indicated by asterisks. The FXa cleavage site is indicated by the arrow and the reactive site residues are labelled. The pyroglutamic acid-blocked *N*-terminus is indicated by *P*-Glu.

potencies toward FXa. The purification of yrATS from large-scale fermentation batches has recently yielded similar results [14]. Accordingly, the blocked and unblocked components were pooled and treated as a single sample for all subsequent analyses. Purification of wild-type and mutant yrATS proteins typically yielded 200–400 µg of purified protein from 1 litre of culture medium. The purity of the final preparations was shown to be at least 90–95 % by reversed-phase h.p.l.c. analysis (Fig. 2b), silver-staining SDS/PAGE (Fig. 3b) and amino-acid-composition analysis (results not shown).

Kinetic analysis of yrATS mutants

The primary sequence of ATS is summarized in Fig. 4. This Figure highlights the FXa-cleavable reactive site located at position 34–35, with arginine and valine corresponding to the P₁ and P₁' residues, respectively. Also illustrated is the two-fold internal sequence identity between the *N*-terminal residues 1–55 and the *C*-terminal residues 56–110. Table 2 shows the kinetic constants for irATS and summarizes the values determined for the wild-type and mutant yrATS proteins. The results demonstrate that wild-type yrATS (R34) is indistinguishable from the irATS previously described [8,9]. The kinetic values shown in Table 2 correspond to the terms outlined in Scheme 2, which describes the two-step inhibitory mechanism utilized by ATS. To determine which amino acid residues are both most potent and acceptable in the P₁ position, the arginine was independently replaced with residues of diminishing polarity. The maintenance of a positively charged residue at this position (R34K) resulted in a 21-fold loss in inhibitory potency; however, this mutant is still a potent FXa inhibitor, as evidenced by a *K_i* value of 1.3 nM. The decreased affinity of this mutant for FXa is accounted for by almost equivalent changes in the association and dissociation rate constants. That is, the *k_{on}* decreases by 4.2-fold and the *k_{off}* increases by 5.6-fold. Replacement of the arginine with an uncharged yet polar residue (R34N) caused a 6000-fold loss in potency, and complete abolition of a charge at the P₁ position (R34L) produced a greater than 16000-fold loss in activity. These results are consistent with the substrate specificity of FXa, namely that substrates with an arginine residue in the P₁ binding site are preferred. Furthermore, these results confirm the mechanism of FXa inhibition proposed for ATS in which it is

Scheme 2

Table 2. Inhibition constants for yrATS mutants

ATS sample	IC ₅₀ (nM)	<i>K_i</i> (final) (pM)	10 ⁷ × <i>k_{on}</i> (M ⁻¹ · s ⁻¹)	10 ⁻⁴ × <i>k_{off}</i> (s ⁻¹)
irATS (R34)*	0.27	43.4 ± 1.4	1.19 ± 0.12	5.2 ± 0.5
yrATS (R34)*	0.26	61.2 ± 1.5	1.23 ± 0.04	7.5 ± 0.2
R34K mutant†	3.90	1280 ± 160	0.29 ± 0.01	41.9 ± 2.0
R34N mutant	1660	ND	ND	ND
R34L mutant	4200	ND	ND	ND
V35I mutant‡	0.74	180 ± 20	2.03 ± 0.21	39.6 ± 6.7
K90V mutant*	0.34	100 ± 2	0.74 ± 0.02	7.5 ± 0.3
K90I mutant*	0.32	86.3 ± 1.6	0.79 ± 0.03	6.8 ± 0.2

* The *K_i* was determined by fitting pre-equilibrated steady-state velocities to Morrison's equation for tight-binding inhibitors, and the *k_{off}* was determined directly by measuring the dissociation rate of pre-equilibrated EI complexes as described in the Materials and methods section; the *k_{on}* was calculated from the relationship: *K_i* = *k_{off}*/*k_{on}*.

† The kinetic constants were determined by analysing slow-binding progress curves as described in the Materials and methods section.

‡ ND, insufficient protein was obtained for a full kinetic analysis of these mutants.

postulated that a cleavable substrate-like reactive site is presented to the enzyme [9].

One of the cleavage sites for FXa in its natural substrate, prothrombin, occurs between residues Arg-322 and Ile-323, which results in the generation of meizothrombin. Since the ATS isoform cloned and utilized in the present studies contains a valine in the corresponding P₁' position, we created a mutant in which the valine was changed to an isoleucine in order to see if a more potent FXa inhibitor could be created (V35I). This

mutant exhibited a 3-fold loss in affinity toward FXa ($K_i = 180$ pM), which was primarily accounted for by an increase in the dissociation rate constant (Table 2). The important contribution of the P_1' residue demonstrated in these studies supports the results obtained previously with a different ATS isoform purified directly from leech salivary-gland extracts [2,3]. That isoform contained a methionine residue at the P_1' position and exhibited a reduced potency of almost 7-fold relative to the ATS isoform containing a valine residue in the P_1' position of the reactive site.

The two-fold internal sequence identity exhibited by ATS (Fig. 4) suggests that the ATS molecule might be folded into separate *N*- and *C*-terminal domains [2,7]. Since mutating the P_1 residue in the *N*-terminal domain abolishes the inhibitory activity of the whole molecule (R34L mutant), the *C*-terminal domain does not appear to represent a functional inhibitory domain. Comparison of the similarity shared between the *N*- and *C*-terminal regions reveals one striking amino acid difference which might account for the inability of the *C*-terminal portion of the molecule to function as an independent inhibitory domain. The position in the *C*-terminal domain which corresponds to the P_1' position in the reactive-site region of the *N*-terminal domain is occupied by a lysine instead of a valine residue (Fig. 4). We have already shown above that substitutions in the P_1' position of ATS significantly affect its affinity for FXa. Consequently, to determine if a second inhibitory domain could be created within the *C*-terminal region of ATS, Lys-90 was mutated to either valine or isoleucine to mimic the reactive site in ATS or the cleavage site in prothrombin respectively. Kinetic analysis of the K90V and K90I proteins revealed dissociation constants modestly higher than wild-type (1.6-fold and 1.4-fold respectively). Sequence analysis of the cleavage products of these mutants after incubation with FXa and purification by reversed-phase h.p.l.c. failed to reveal any detectable cleavage products between residues Arg-Val or Arg-Ile at position 90 (results not shown). The only detectable cleavage product was that with the reactive site hydrolysed at position Arg-34. Gel analysis of the cleavage products also failed to detect any cleavage fragments other than the product resulting from cleavage of the reactive site (Fig. 5b). These results therefore demonstrate that, although the *N*- and *C*-terminal domains of ATS share significant amino acid sequence similarity, the *C*-terminal domain is not folded into an orientation which facilitates a strong binding interaction with FXa. However, we cannot exclude the possibility that the *C*-terminal domain exhibits extremely weak inhibitory activity (i.e. K_i in the high micromolar range), since this level of inhibition would be masked by the *N*-terminal reactive-site domain.

Cleavage analysis of yrATS mutants by FXa

We have shown previously that ATS inhibits FXa according to a common mechanism used by most small protein inhibitors of serine proteinases [9]. This mechanism is characterized by the slow partial hydrolysis of the reactive-site peptide bond, leading to an equilibrium approaching unity between the native and hydrolysed forms of the inhibitor [10–12]. We analysed the cleavage products of each yrATS mutant protein after prolonged incubation with FXa to determine if the reduced inhibitory potencies manifest by the various mutants resulted from an interruption in this mechanism. As shown in Fig. 5(a), the decreasing inhibitory potency of the mutants in the P_1 position correlates with the reduced ability of FXa to hydrolyse the reactive-site peptide bond. Consonant with its relatively high inhibitory potency, the R34K mutant reaches a hydrolysis equilibrium similar to wild-type yrATS. In contrast, the R34N and R34L mutants fail to serve as limited proteolytic substrates for FXa. Fig. 5(b) reveals that the P_1' mutant, V35I, exhibits a

Fig. 5. FXa cleavage analysis of yrATS mutants

Each purified yrATS protein was incubated for 24 h at 37 °C in the presence (+) or absence (–) of FXa and then analysed on 10–20% polyacrylamide/SDS/Tricine gels under reducing conditions. Proteins were revealed by staining with Coomassie Blue. (a) Represents yrATS mutants in the P_1 position, and (b) represents yrATS mutants in the P_1' position and K90 position. Lane M consists of M_r markers, and lane C contains FXa incubated alone for 24 h.

hydrolysis equilibrium shifted modestly toward the hydrolysed form of the molecule. This is consistent with the 3-fold reduction in inhibitory potency of this mutant and suggests that the loss in potency reflects the reduced affinity between FXa and the hydrolysed inhibitor. As mentioned above, the two mutants in the *C*-terminal half of the molecule, K90V and K90I, display hydrolysis patterns and equilibrium ratios indistinguishable from those of the wild-type.

Analysis of yrATS mutants in APTT clotting assay

Under physiological conditions, solution-phase FXa does not efficiently catalyse the formation of thrombin. Instead, FXa associates with its cofactor, Factor Va, and Ca^{2+} on the surface

Fig. 6. Analysis of yrATS mutants in APTT clotting assay

Each mutant preparation was analysed in a human plasma-based clotting assay as described in the Materials and methods section. ○, yrATS wild type; ▲, R34K mutant; ■, R34N mutant; ▼, R34L mutant; □, V35I mutant; ◇, K90V mutant and △, K90I mutant. The maximum clotting time was 150 s.

of activated platelets to yield an active prothrombinase complex. To investigate whether the effects of the various yrATS mutations observed in the purified 'in vitro' system would also occur when FXa is assembled into the prothrombinase complex, the comparative potencies of the mutants were determined in a standard APTT plasma-based clotting assay. The results of this analysis are summarized in Fig. 6. The relative inhibitory profiles of the mutants spanning the P₁ and P₁' positions mirror the results obtained in the amidolytic FXa assay. In contrast, the two mutants at residue 90, K90V and K90I, exhibit considerably reduced potencies approaching that of R34K in this assay, whereas they were only minimally less potent than wild-type yrATS in the chromogenic FXa assay. These results suggest that a positively charged amino acid at residue 90 may play a significant role in stabilizing the interaction between ATS and FXa when FXa is assembled into the prothrombinase complex. The highly conserved nature of the lysine residue in this position in four ATS isoforms identified from the leech *Haementeria officinalis* [2,7,8] as well as a single ATS isoform purified from a related leech, *H. ghilianii* [25,26], provides supporting evidence that this residue may have an important function.

Conclusion

In the present study we have provided additional evidence demonstrating that ATS inhibits FXa according to a standard mechanism in which a surface-exposed reactive site interacts with the enzyme in a substrate-like manner. This mechanism can be contrasted with the mechanism of inhibition utilized by the serpin (Serine-proteinase-inhibitor) family of proteinase inhibitors [27,28], as well as the potent leech-derived thrombin inhibitor hirudin [29–31]. Our results suggest that the C-terminal domain of ATS is not in the proper conformation to expose a second inhibitory reactive site; however, the conserved lysine residue at position 90 in the C-terminal half of ATS appears to play an important role in the inhibition of FXa assembled in a prothrombinase complex. Future studies that elucidate the structural properties of ATS which account for its selectivity toward FXa should help in the design of rational inhibitors of this important coagulation enzyme.

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