Identification and Characterization of Variants of Tick Anticoagulant Peptide with Increased Inhibitory Potency toward Human Factor Xa

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ABSTRACT: Tick anticoagulant peptide (TAP) is a specific and potent inhibitor of factor Xa (fXa), a central enzyme in the blood clotting cascade. As such, TAP is a potential antithrombotic agent. Site-directed mutagenesis studies were undertaken to determine the feasibility of increasing the inhibitory potency of TAP toward fXa. The amino acid substitutions Tyr-1 to Trp (Y1W) and Asp-10 to Arg (D10R) increased inhibitory potency toward human fXa by 2.5- and 4-fold, respectively. The increased inhibitory potency reflected a decrease in the rate constant for dissociation of the final fXa−TAP inhibitory complex. The double mutant, Y1W/D10R, exhibited an inhibition constant of 10 pM, a 37-fold enhancement of inhibitory potency toward human fXa. The improvement in inhibitory potency was less pronounced (12-fold) with dog fXa wherein K_is of 220 and 18 pM were observed for wild-type TAP and the double mutant, respectively. Mutation of Tyr-1 to Glu (Y1E) generated a weaker inhibitor (K_i = 2 nM) that bound human fXa more slowly. However, no change in inhibitory potency toward human fXa was observed when Tyr-1 was replaced by Phe. Taken together, these observations are consistent with the view that a hydrophobic amino acid at the N-terminus of TAP may be a determinant of inhibitory potency. Decreases by 3−4 orders of magnitude in inhibitory potency were noted upon mutation of Asn-2 and Leu-4 of TAP, further implicating the N-terminal domain as an important determinant of inhibitory potency. A peptide, TAP(1/9), corresponding to the first nine residues of TAP (with a Cys-5 to Ala replacement) was a competitive inhibitor of fXa-catalyzed hydrolysis of a small chromogenic substrate (K_i = 100 μM). This result suggests that the N-terminal domain of TAP interacts with the active site of fXa.

The serine protease factor Xa (fXa)[1] is an essential enzyme in the blood coagulation cascade that is positioned at the intersection of the intrinsic and extrinsic pathways. FXa, the protein cofactor Va, calcium ion, and an appropriate phospholipid membrane, such as a platelet surface, comprise the prothrombinase complex which functions as the physiological system that activates prothrombin to thrombin *in vivo*. The resulting thrombin plays many important physiological roles including cleavage of the soluble plasma protein fibrinogen to fibrin, the insoluble matrix of blood clots. A recent report (Eisenberg *et al.*, 1993) suggests that clot-bound fXa is important for procoagulant activity of arterial thrombi and whole-blood clots. This activity is resistant to inhibition by antithrombin III-dependent inhibitors such as heparin. The direct fXa inhibitor tick anticoagulant peptide (TAP), however, could prevent this procoagulant activity. TAP from the tick *Ornithodoros moubata*, a peptide comprising 60 amino acids and three disulfide bond linkages, is a potent inhibitor of fXa (Waxman et al., 1990). Recombinant TAP has been successfully expressed in yeast *Saccharomyces cerevisiae* (Neeper et al., 1990) and shown to exhibit identical inhibitory potency toward fXa to that of native TAP. It is a specific, reversible, tight-binding, and competitive inhibitor of fXa (Jordan et al., 1990) that exhibits therapeutic efficacy in animal models of thrombosis (Vlasuk, 1993). Further kinetic studies (Jordan et al., 1992) using stopped-flow spectrofluorometry demonstrated that the interaction of TAP with human fXa proceeds via a two-step pathway wherein a rapidly formed initial inhibitory complex, E:I, slowly rearranges to a more stable complex, E:I* (Scheme 1), and that TAP interacts with fXa at both the active site and an exosite distinct from the active site.

Scheme 1

$$E + I \underset{k_{-2}}{\overset{K_i'}{\rightleftharpoons}} E{:}I \overset{k_2}{\rightleftharpoons} E{:}I^*$$

A study using site-directed mutagenesis to probe the role of the ionic residues of TAP (Dunwiddie et al., 1992) identified Arg-3 as an important determinant of inhibitory potency. In this study it was shown that mutation of Arg-3 to a neutral amino acid (R3N, Arg-3 to Asn) resulted in a 10,000-fold decrease in inhibitory potency toward human fXa, whereas none of the other charged to neutral amino acid replacements produced more than a 4-fold change in inhibitory potency. In addition, synthetic TAP variants in which the first four residues were systematically truncated exhibited a dramatic decrease of inhibitory potency. These

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[1] Abbreviations: Chromzyme TH, tosyl-Gly-Pro-Arg-*p*-nitroanilide; DEGR-Xa, active site histidine derivative of factor Xa obtained by reaction of factor Xa with dansyl-L-Glu-Gly-L-argininyl chloromethyl ketone; fXa, factor Xa; FMGB, fluorescein mono-*p*-guanidinobenzoate; IEGR-AMC, 7-[*N*-[(*tert*-butyloxycarbonyl)-L-Ile-L-Glu-Gly-L-Arg]-amido]-4-methylcoumarin; PEG, poly(ethylene glycol); Spectrozyme Xa, (methoxycarbonyl)-D-(cyclohexyl-Gly)-Arg-*p*-nitroanilide; SBTI, soybean trypsin inhibitor; TAP, tick anticoagulant peptide; TAP (1/9); Tyr-Asn-Arg-Leu-Ala-Ile-Lys-Pro-Arg; TBS, Tris-buffered saline; TBSP, Tris-buffered saline with PEG 8000.

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results of Dunwiddie et al. (1992) suggest that the amino-terminal region of TAP interacts with fXa.

In the present study, we demonstrate that the inhibitory potency of TAP toward human fXa can be markedly increased by site-directed mutagenesis. We also present evidence suggesting that the N-terminal domain of TAP interacts with the active site of fXa.

EXPERIMENTAL PROCEDURES

Materials. The fluorogenic substrate 7-[*N*-(*t*-Boc-Ile-Glu-Gly-Arg)amido]-4-methylcoumarin (IEGR-AMC) was purchased from Sigma, dissolved in Millipore purified water, and filtered (0.2 μm). Its concentration was determined in water using $\epsilon_{325nm} = 1.72 \times 10^4$ M^{-1} cm^{-1}. The chromogenic substrate Spectrozyme Xa was purchased from American Diagnostic, Inc., and prepared as recommended. Chromzyme TH was purchased from Sigma. The concentrations of Spectrozyme Xa and Chromzyme TH were determined using $\epsilon_{342nm} = 8.27 \times 10^3$ M^{-1} cm^{-1} (Lottenberg et al., 1981).

Human fXa was prepared from human factor X (Haematologic Technologies) by the method of Bock *et al.* (1989). FXa concentrations were determined using $\epsilon_{280nm} = 1.16$ $(mg/mL)^{-1}$ and a molecular weight of 46 000 (Di Scipio et al., 1977a,b). The fXa was homogeneous (>90% α-fXa) as judged by sodium dodecyl sulfate gel electrophoresis under reducing conditions in the Laemmli (1970) buffer system. Active site concentrations were determined by titration with fluorescein mono-*p*-guanidinobenzoate (FMGB) using the method of Bock et al. (1989).

Construction of TAP Variants. TAP variants were constructed according to the procedure described by Dunwiddie *et al.* (1992) except for the double mutant Y1W/D10R. The oligonucleotide primer sequences for the mutants were 5′-CAG-ACG-GTT-ATC-CCT-TTT-ATC (Y1D), CAG-ACG-GTT-TTC-CCT-TTT-ATC (Y1E), CAG-ACG-GTT-AAA-CCT-TTT-ATC (Y1F), CAG-ACG-GTT-CCA-CCT-TTT-ATC (Y1W), GCA-CAG-ACG-GGC-GTA-CCT-TTT (N2A), TTT-GAT-GCA-CCT-ACG-GTT-GTA (L4R), ACG-CGG-TTT-TCT-GCA-CAG-ACG (I6R), GTC-ACG-CCC-TTT-GAT-GCA-CAG (K7R), and GTC-GAT-CCA-GCG-ACG-CGG-TTT (D10R). The gene for the double mutant, Y1W/D10R, was produced by polymerase chain reaction (PCR) using the single mutant D10R DNA as template. A sense oligonucleotide, 5′-TGG-AAC-CGT-CTG-TGC-ATC, and a 3′ complementary oligonucleotide, 5′-ACT-GGA-TCC-AAT-TCA-ACG-TTA, were annealed with D10R template and amplified. The PCR product was digested with *Bam*HI to generate a cohesive 3′-end and ligated into the *Sph*I (also blunted by T4 DNA polymerase) and *Bgl*II sites of the original TAP vector (Neeper et al., 1990). The double-stranded DNA sequences of all mutants were confirmed using Sequenase TM (U.S. Biochemical).

Isolation of TAP Variants. TAP variants were isolated from fermentation broth of yeast strain DMY6 containing mutant plasmids. Yeast broth (200−1000 mL) was filtered using a filter unit with a 0.8-μm membrane (Nalgene) and diluted with 50 mM acetate buffer, pH 4.0. The filtrate was loaded onto an SP-Sepharose fast-flow column (Pharmacia) and eluted with a gradient of 0 to 1.2 M NaCl in acetate buffer. FXa inhibitory fractions were concentrated using an Amicon apparatus with a YM3 membrane to an appropriate volume for reversed-phase HPLC (Waters 600E system connected with a Waters 994 photodiode-array detector). A C_{18} semiprep column (Vydac) was used for the final purification of TAP variants. The gradient for HPLC was 100% water containing 0.1% trifluoroacetic acid (TFA) (buffer A) for 5 min, followed by a gradient of 0 to 25% acetonitrile containing 0.1% TFA (buffer B) in 5 min and then a second gradient of 25 to 40% buffer B in 30 min. The flow rate was 2 mL/min, and the absorbance of the effluent stream was monitored at 280 nm. Fractions with fXa inhibitory activity (retention times, 26−31 min) were pooled and lyophilized to dryness. The resulting TAP variants were dissolved in water and characterized. The concentrations of TAP were determined by absorbance at 280 nm using $\epsilon_{280} = 1.78 \times 10^4$ M^{-1} cm^{-1} (Jordan et al., 1992) with the exception of variants containing an amino acid change at residue 1 (tyrosine). For mutant Y1W and the double mutant Y1W/D10R, an extinction coefficient of 2.22×10^4 M^{-1} cm^{-1} was used; for mutants Y1D, Y1E, Y1F, and Y1R, a value of 1.65×10^4 M^{-1} cm^{-1} was used. Extinction coefficients were calculated from the amino acid composition of the TAP variant using the method of Gill and von Hippel (1989). The concentrations of stock solutions of all TAP variants were confirmed by quantitative amino acid analysis and were within 10% of the values expected from the absorbance. The sequence of the first 15 residues of the double mutant was confirmed by N-terminal sequencing.

FXa Assay. Enzyme activity was determined by monitoring the increase in absorbance at 405 nm that is due to release of *p*-nitroaniline ($\epsilon_{405} = 9920$ M^{-1} cm^{-1}) upon hydrolysis of the chromogenic substrate, Spectrozyme Xa. FXa, 0.5−1.5 nM, was preincubated with various concentrations of TAP variants (0.1−10 nM) in TBSP buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, and 0.1% PEG-8000 in a PEG-20000 precoated microtiter plate at room temperature for >4 h. Spectrozyme Xa (final concentration, 200 μM) was then added, and the hydrolysis rate was measured. Morrison's equation for tight-binding inhibitors was used to calculate the inhibition from the dependence of hydrolysis rate on the concentration of TAP at a fixed concentration of fXa (Morrison, 1969).

For the weaker binding TAP variants, Y1R, N2A, N2D, N2R, and L4R, 15 μM Chromzyme TH (K_m = 175 μM; therefore, [S] $\ll$ K_m) was used to determine the inhibition constants from a linear least squares fit of the data to eq 1,

$$V_0/V_i = 1 + [TAP]/K_i \quad (1)$$

where V_0 and V_i are fXa-mediated hydrolysis velocities in the absence and presence of TAP variants, respectively. Because Chromzyme TH is a substrate of thrombin, recombinant hirudin (r-hirudin) was added to block any hydrolysis of substrate by contaminating thrombin (<0.8%). All reaction mixtures were preincubated at room temperature in TBSP buffer with 2 nM human fXa containing 0.1 nM r-hirudin for 4 h.

Determinations of Second-Order Association Rate Constants. These determinations were performed using an Applied Photophysics SX.17MW sequential stopped-flow spectrofluorometer interfaced with an Archimedes 420/I computer. All assays were performed in TBSP buffer at 25

°C. Association rates were measured under pseudo-first-order conditions. TAP variant (100−300 nM initial concentrations) was mixed with the substrate, IEGR-AMC (100 μM initial concentration), prior to being reacted with an equal volume of a solution of human fXa (5−10 nM initial concentrations). Hydrolysis of IEGR-AMC was monitored fluorometrically with excitation at 380 nm using a 400-nm Oriel cutoff fluorescence emission block. The values of observed pseudo-first-order rate constants (k_{obs}) were derived from nonlinear regression fits of the average of 4−6 progress curves to eq 2 as described by Williams and Morrison (1979), Cha (1975), and Morrison and Walsh (1988).

$$F = F_0 + V_s t + (V_0 - V_s)(1 - e^{-k_{obs}t})/k_{obs} \quad (2)$$

F_0, F, V_0, and V_s represent the initial fluorescence, the fluorescence at time t, and the initial and final rates of change of fluorescence, respectively. Because the substrate concentration (50 μM, final concentration) was below $0.1K_m$ (K_m = 750 μM; Jordan et al., 1992), k_{obs} is related to the second-order association rate constant, k_{on}, by eq 3 in the range of TAP concentrations where k_{obs} is a linear function of the concentration of TAP.

$$k_{obs} = k_{on}[TAP] + k_{off} \quad (3)$$

Determination of Kinetic Constants for Scheme 1. Values of k_{obs} were determined as described above by reacting a solution containing TAP variant (1−400 μM initial concentrations) and substrate, IEGR-AMC (100 μM initial concentration), with an equal volume of a solution of fXa (0.2−4 μM initial concentration). Equation 2 was used to determine k_{obs}. The fit of the dependence of k_{obs} on the TAP concentration to eq 4 was used to determine the values of k_2 and K_i' of Scheme 1.

$$k_{obs} = k_{-2} + k_2[TAP]/\{K_i' + [TAP]\} \quad (4)$$

Values of k_{-2} were determined from the relationship $k_{-2} = k_2 (K_i' - K_i)/K_i$. When [TAP] < K_i', eq 4 reduces to eq 3 with $k_{off} = k_{-2}$, and $k_{on} = k_2/K_i'$.

Isolation and Activation of Dog FXa. Dog factor X was purified from citrated dog plasma (purchased from PEL-FREEZ) by barium chloride precipitation and DEAE-Sepharose anion-exchange chromatography using methods previously described by Bock for purification of human factor X. Dog factor X was activated by Russells' viper venom by the procedure used to activate human fX, and purified by use of a soy bean trypsin inhibitor (SBTI) affinity column (Bock et al., 1989). The concentration of dog fXa was determined using FMGB as titrant.

Inhibition of FXa by TAP(1/9). The effect of TAP(1/9), a peptide containing amino acid residues 1−9 of TAP with a replacement of alanine at Cys-5, on enzyme activity was determined by monitoring the increase in absorbance at 405 nm associated with fXa-mediated hydrolysis of the chromogenic substrate Spectrozyme Xa. FXa (final concentration, 0.5 nM) was added to various concentrations (100−420 μM) of TAP(1/9) with various concentrations of substrate (25−500 μM) at 25 °C in TBSP buffer on a PEG-20000 precoated microtiter plate at room temperature. A double-reciprocal plot ($1/V$ vs $1/[S]$) was used to obtain the apparent K_m' as a function of the concentration of inhibitor. The competitive inhibition constant K_i was derived from the fit of the linear dependence of K_m' on inhibitor concentration to eq 5.

$$K_m'/K_m = 1 + [TAP(1/9)]/K_i \quad (5)$$

Table 1: Equilibrium and Rate Constants for Inhibition of Human FXa by TAP Variants[a]

TAP variant	K_i (nM)	rel potency	k_{on} (μM^{-1} s^{-1})	rel k_{on}
wild type	0.37 ± 0.05	1.00	1.8	1.00
Y1W	0.15 ± 0.01	2.5	1.9	1.03
Y1D	2.3 ± 0.4	0.17	0.77	0.43
Y1E	2.0 ± 0.2	0.19	0.67	0.37
Y1F	0.34 ± 0.03	1.10	1.7	0.94
Y1R	35 ± 5	0.011		
N2A	500 ± 30	0.000 71		
N2D	1100 ± 100	0.000 34		
N2R	8900 ± 400	0.000 042		
L4R	860 ± 20	0.000 43		
I6R	0.61 ± 0.07	0.65	1.2	0.67
K7R	0.54 ± 0.10	0.73	1.2	0.67
D10R	0.10 ± 0.03	3.90	1.6	0.89

[a] All constants were determined at pH 7.4 in buffer containing 0.05 M Tris, 0.15 M NaCl, and 0.1% PEG-8000 at room temperature (K_i) or 25 °C (k_{on}).

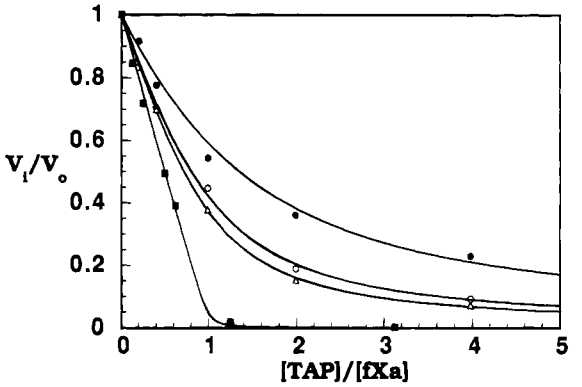

FIGURE 1: Inhibition of human fXa by TAP variants. Preincubation mixtures containing (final concentrations) fXa (1.0 nM) and TAP variants (0.2−10 nM) in TBSP reaction buffer were incubated at room temperature in a PEG 20000-precoated microtiter plate for 4 h. Activities (V_0) of free enzyme were determined from the increase of absorbance upon fXa-mediated hydrolysis of a small chromogenic substrate (Spectrozyme Xa, 200 μM) at 405 nm. The solid line, which reflects a nonlinear least squares fit of the data to Morrison's equation with a fixed total concentration of fXa, yielded the reported values of K_i. Wild-type TAP (●), Y1W (○), D10R (△) and Y1W/D10R (■).

RESULTS

Inhibition constants for TAP variants (Table 1) were determined by analyzing the effect of TAP on fXa-catalyzed hydrolysis of a small chromogenic substrate (Figure 1). For these determinations fXa and the TAP variants were preincubated for sufficient time to attain equilibrium before addition of a small volume of substrate solution. Replacement of the N-terminal Tyr with either cationic or anionic amino acids (variants Y1R, Y1D, and Y1E) significantly decreased inhibitory potency (Table 1). When the hydrophobic character of the N-terminal residue of TAP was increased by replacement of Tyr-1 with a Trp, Y1W, a 2.5-fold enhancement in inhibitory potency toward human fXa was observed. No change in inhibitory potency was observed, however, for the phenylalanine variant (Y1F). Taken together, these observations are consistent with the

Table 2: Equilibrium and Rate Constants for Inhibition of Human and Dog FXa by TAP and Y1W/D10R[a]

	human factor Xa			dog factor Xa		
	K_i (pM)	k_{on} (μM^{-1} s^{-1})	10^{-3} k_{off}[b] (s^{-1})	K_i (pM)	k_{on} (μM^{-1} s^{-1})	10^{-3} k_{off}[b] (s^{-1})
wild type	370 ± 50	1.8 ± 0.2	0.67	220 ± 10	3.3 ± 0.3	0.73
Y1W/D10R	10 ± 3	1.8 ± 0.3	0.018	18 ± 3	3.1 ± 0.1	0.056

[a] All constants were determined at pH 7.4 in buffer containing 0.05 M Tris, 0.15 M NaCl, and 0.1% PEG-8000 at room temperature (K_i) or 25 °C (k_{on}). [b] k_{off} was determined from the relationship $k_{off} = k_{on}K_i$.

existence of a hydrophobic interaction between the N-terminal residue of TAP and fXa.

Replacement of the anionic Asp at position 10 with an Arg yielded a variant (D10R, K_i = 100 pM) that exhibited a 4-fold increase in inhibitory potency relative to that of wild-type TAP (K_i = 370 pM). The enhancement in potency of the two single variants, Y1W and D10R, prompted us to create the double variant Y1W/D10R. The structure of Y1W/D10R was confirmed by DNA sequencing of the cDNA used to express Y1W/D10R, quantitative amino acid analysis, and amino-terminal sequencing of the protein. Y1W/D10R was a more potent inhibitor (K_i = 10 ± 3 pM) than either single mutant (Table 2 and Figure 1). The 37-fold increase in the inhibitory potency is larger than the 10-fold additive effect expected for the combined effects of the Y1W (2.5-fold) and D10R (4-fold) replacements. Extension of the N-terminal α-amino group of Y1W/D10R with an additional amino acid (glycine) did not alter the inhibitory potency (data not shown). As illustrated in Figure 1, Y1W/D10R is an excellent titrant of fXa. Examination of the data in Table 1 reveals that the less potent variants, Y1E and Y1D, bind fXa more slowly than does wild-type TAP. The observation that the rate constants for association of Y1W, D10R, and Y1W/D10R with fXa are nearly identical to that of wild-type TAP indicates that the enhanced affinity of these more potent variants is due to their slower dissociation from fXa.

The observation that the inhibitory potency of certain fXa inhibitors varies with the species of origin of fXa (Tidwell et al., 1980; Ishihara et al., 1993) prompted us to characterize the interaction of a wild-type TAP and Y1W/D10R with fXa from a nonhuman species. Purified dog fXa showed the same K_m and k_{cat} as human fXa on fXa-mediated hydrolysis of the chromogenic substrate, Spectrozyme Xa (data not shown). TAP inhibited dog fXa (K_i = 220 ± 10 pM) 1.7-fold more potently than it inhibited human fXa. The increase in inhibitory potency toward dog fXa appears to be a consequence of a 1.8-fold larger second-order rate constant for association of wild-type TAP with dog fXa (Table 2). The data listed in Table 2 indicate that Y1W/D10R is 12-fold more potent than wild-type TAP in inhibiting dog fXa. Thus, the inhibitory potency of wild-type TAP and the enhancement of inhibitory potency exhibited by Y1W/D10R are species-dependent. As in the case of human fXa, the increased inhibitory potency (12-fold in the case of the dog fXa) is due to a slower rate of dissociation of the Y1W/D10R from fXa (Table 2).

The interaction of TAP with fXa was shown to proceed via a two-step reaction pathway as depicted in Scheme 1, wherein a rapidly formed initial complex, E:I, rearranges in a rate-determining step to a more stable inhibitory complex, E:I* (Jordan et al., 1992). The TAP variants Y1W and Y1E exhibited kinetic evidence for a two-step pathway like that observed for wild-type TAP (Figure 2). Values for the dissociation constants of the initial complex (K_i') and the slow rearrangement rate constants (k_2 and k_{-2}) are compared for TAP, Y1W, and Y1E in Table 3. Y1W differs from wild-type TAP in that the rate constant k_{-2} is decreased. The slower reacting mutant Y1E , however, shows a 3.5-fold larger dissociation constant (240 μM) for the initial complex, E:I, than does wild-type TAP (68 μM), but retains similar values for the rate constant k_2. These results suggest that the slower reaction rate (k_{on}) of Y1E with fXa is due to the formation of a weaker initial complex. The limited solubility of the variants D10R and Y1W/D10R (<50 μM in reaction buffer) precluded determination of K_i' and k_2 for those variants.

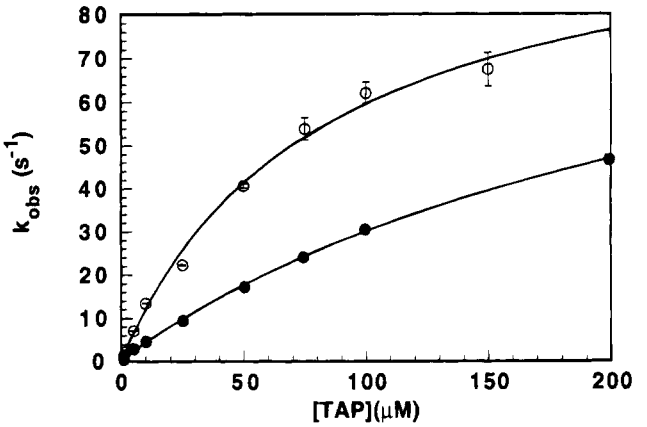

FIGURE 2: Dependence of the observed pseudo-first-order association rate constant (k_{obs}) for inhibition of fXa by Y1W (○) and Y1E (●). The error bars reflect the standard deviation of the plotted values of k_{obs}. The best fit of the data to eq 4 (solid line) yields values of 79 ± 6 μM and 107 ± 6 s^{-1} for K_i' and k_2, respectively, for Y1W and values of 240 ± 20 μM and 104 ± 5 s^{-1} for K_i' and k_2, respectively, for Y1E.

Table 3: Characterization of the Reaction Pathway for Inhibition of FXa by TAP Variants[a]

TAP variant	K_i (nM)	K_i' (μM)	k_2 (s^{-1})	k_{-2} (s^{-1})[b]
wild type	0.37 ± 0.05	68 ± 6	123 ± 5	6.7×10^{-4}
Y1W	0.15 ± 0.01	79 ± 6	107 ± 6	2.0×10^{-4}
Y1E	2.0 ± 0.2	240 ± 20	104 ± 5	8.6×10^{-4}

[a] All constants were determined at pH 7.4 in buffer containing 0.05 M Tris, 0.15 M NaCl, and 0.1% PEG-8000 at room temperature (K_i) or 25 °C (K_i' and k_2). [b] k_{-2} was evaluated from the relationship $k_{-2} = k_2K_i/(K_i' - K_i)$.

To further characterize the interaction of the amino terminus of TAP with fXa, a peptide, TAP(1/9), derived from the first nine residues of the amino-terminal sequence of TAP with a Cys-5 to alanine replacement was prepared. This peptide exhibited competitive inhibition of the human fXa-mediated hydrolysis of a small substrate with a K_i of 100 μM (Figure 3). The competitive inhibition of human fXa by the peptide TAP(1/9) is consistent with an interaction of the amino terminus of TAP with the active site of fXa.

DISCUSSION

In many respects, the interactions of TAP with fXa appear to be analogous to those of hirudin with thrombin. Hirudin binds with its N-terminal three residues in the active site

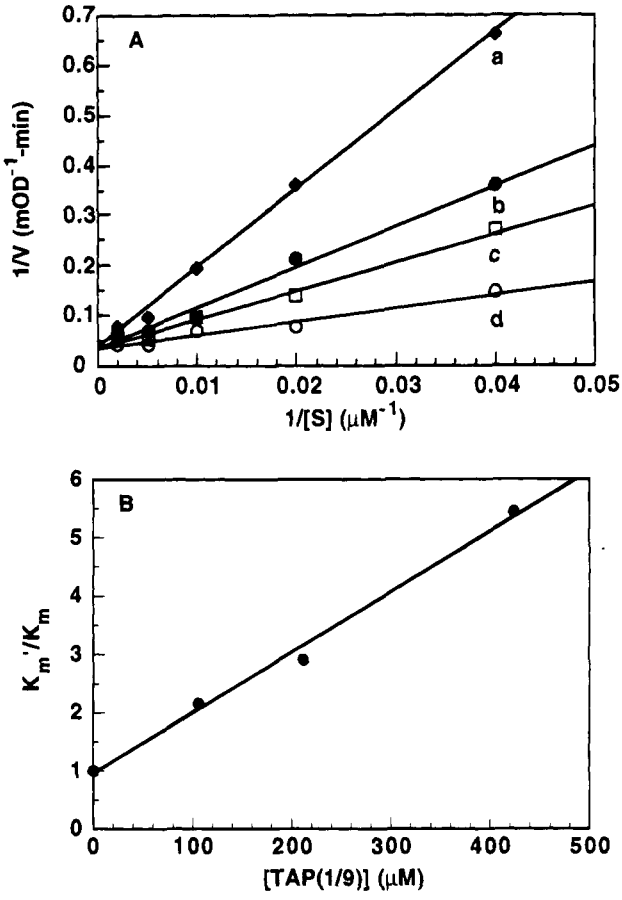

FIGURE 3: (A) Competitive inhibition of fXa-catalyzed hydrolysis of Spectrozyme Xa by TAP (1/9). (B) Determination of the value of K_i for inhibition of fXa catalysis by TAP (1/9). The plotted values of K_m and K_m' were determined from the slope:intercept ratio of the plots in panel A. The value of K_i was determined from a linear least squares fit of the data to eq 5.

cleft of thrombin (Rydel et al., 1990; Grutter et al., 1990). Like TAP, hirudin contains a hydrophobic N-terminal amino acid (valine or isoleucine) that appears to be an important determinant of inhibitory potency. Together with the conserved Tyr-3, this apolar residue makes hydrophobic contacts with thrombin as shown by X-ray diffraction analysis. The importance of the hydrophobic N-terminal residue was demonstrated by mutagenesis studies that showed that substitution of the N-terminal residue of hirudin with a polar amino acid (lysine, glutamate, and serine) decreases inhibitory potency by 1200-fold, whereas replacement of the N-terminal residue with other hydrophobic amino acids (e.g., phenylalanine or leucine) results in modest or negligible decreases in inhibitory potency (Wallace et al., 1989; Stone et al., 1990).

The observation that a peptide homologous to the amino-terminal domain of TAP is a competitive inhibitor of fXa-catalyzed small substrate hydrolysis suggests that the N-terminal domain of TAP, like that of hirudin, interacts near the active site of its target protease. In this regard it is interesting to note that the TAP variants N2D and N2R show a ~10,000-fold decrease in inhibitory potency and bind to fXa with an affinity (K_i = 1.1−8.9 μM, Table 1) that is similar to that observed for the interaction of wild-type TAP with the active site blocked fXa derivative DEGR-Xa (K_d = 5.9 μM). The importance of hydrophobicity at the amino-terminal residue of TAP was further demonstrated by the tryptophan mutation (Y1W) that increased the inhibitory potency, as well as by the marked loss of inhibitory potency observed when the terminal amino acid residue was substituted with a polar amino acid (Y1D, Y1E, and Y1R). Whereas this effect of polar substitution at the amino terminus is qualitatively similar to that observed with hirudin, there are noteworthy differences between the interactions of the N-terminal domain of TAP and hirudin with their target proteases. Instead of a hydrophobic amino acid at residue 3, TAP contains a cationic amino acid, arginine, that is essential for inhibition of fXa. The α-amino group of the N-terminus of hirudin interacts with the active site Ser-195 and vicinal residues in thrombin (Rydel et al., 1990; Grutter et al., 1990). Extension of this α-amino group of hirudin by a single glycine residue or simple acetylation results in a marked decrease in the inhibitory potency (10^3−10^4-fold) toward thrombin (Wallace et al., 1989; Stone et al., 1990), whereas extension of the amino terminus of Y1W/D10R with a single glycine appears to have little effect on inhibitory potency.

The observation (Jordan *et al.*, 1992) that TAP binds to fXa when the active site of the enzyme is blocked either by covalent modification of the active site His or by occupancy of the P1 specificity pocket by *p*-aminobenzamidine (P) suggests that TAP interacts with fXa at both the active site and an exosite distinct from the active site. The change in K_i' observed with the TAP variant Y1E, together with the observation that TAP (1/9) is a competitive inhibitor of fXa, suggests that the N-terminal domain of TAP interacts with the active site of fXa during the first step of the reaction pathway (Scheme 1) for formation of the final inhibitor complex E:I*. It is important to note that the involvement of active site interactions during the first step of the reaction pathway does not preclude the involvement of active site as well as exosite interactions during both phases of the reaction. The observation that mutations in the N-terminal domain of TAP affect both K_i and k_{-2} suggests that the N-terminal region of TAP may indeed interact with fXa during both steps of the reaction pathway. It is interesting to note that in the reaction of TAP with the fXa:P complex, a fXa:TAP complex corresponding to E:I of Scheme 1 is formed upon displacement of *p*-aminobenzamidine (P) from the active site of the TAP:fXa:P ternary complex (Jordan *et al.*, 1992). The resulting binary E:I complex then rearranges to the final stable inhibitory complex E:I*. As pointed out by Jordan *et al.* (1992), the rate constant for rearrangement of E:I to the final inhibitory complex E:I* is about 40 times smaller than the corresponding rate constant for formation of the final inhibitory complex E:I* from E:I when E:I is generated upon reaction of uncomplexed fXa with TAP. This observation indicates that the pathway for formation of the common end product E:I* is altered by blocking the P_1 specificity pocket of fXa (i.e., the structure of E:I, but not that of E:I*, is pathway dependent).

It is interesting to consider how the active site and exosite interactions contribute to the overall affinity of TAP for fXa. Let us assume that the free energy for the interactions of TAP with the active site of fXa can be estimated from the K_i (100 μM) observed with TAP (1/9), and that the free energy for the interaction of TAP with the fXa exosite can be estimated from the value of 5.9 μM for the dissociation constant of the DEGR-Xa:TAP complex (Jordan et al., 1992). If we add these free energies and use a factor of 4.6 to correct for the favorable entropic proximity effect (Koshland, 1962)

for forming the second complex after the first complex is formed, we obtain an estimate of 130 pM ($100 \times 10^{-6} \times 5.9 \times 10^{-6}/4.6$) for the equilibrium constant for dissociation of TAP from fXa. This value is only 3-fold lower than the value of 370 pM observed for the K_i for inhibition of fXa by wild-type TAP. Considering the structural limitations of the models used for the active site and exosite interactions, the correspondence is tolerable between the K_i estimated from the behavior of the models and the observed K_i for wild-type TAP.

Although we have demonstrated that we can increase the inhibitory potency of wild-type TAP for human fXa, further studies are necessary to determine the extent to which the 37-fold increase in potency increases the efficacy of Y1W/D10R as an antithrombotic therapeutic agent.

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