Tick Anticoagulant Peptide: Kinetic Analysis of the Recombinant Inhibitor with Blood Coagulation Factor X_a

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ABSTRACT: Tick anticoagulant peptide (TAP) is a 60 amino acid protein which is a highly specific inhibitor of human blood coagulation factor X_a (fX_a) isolated from the tick *Ornithodoros moubata* [Waxman, L., Smith, D. E., Arcuri, K. E., & Vlasuk, G. P. (1990) *Science 248*, 593–596]. Due to the limited quantities of native TAP, a recombinant version of TAP produced in *Saccharomyces cerevisiae* was used for a detailed kinetic analysis of the inhibition interaction with human fX_a. rTAP was determined to be a reversible, slow, tight-binding inhibitor of fX_a, displaying a competitive type of inhibition. The binding of rTAP to fX_a is stoichiometric with a dissociation constant of $(1.8 \pm 0.02) \times 10^{-10}$ M, a calculated association rate constant of $(2.85 \pm 0.07) \times 10^6$ M^{-1} s^{-1}, and a dissociation rate constant of $(0.554 \pm 0.178) \times 10^{-3}$ s^{-1}. Binding studies show that ^{35}S-rTAP binds only to fX_a and not to DFP-treated fX_a or zymogen factor X, which suggests the active site of fX_a is required for rTAP inhibition. That rTAP is a unique serine proteinase inhibitor is suggested both by its high specificity for its target enzyme, fX_a, and also by its unique structure.

Blood coagulation involves a series of enzyme-catalyzed steps that leads to a burst of thrombin formation which ultimately results in the generation of a stable fibrin-based clot. The activated serine protease factor X_a (fX_a)[1] plays a pivotal role in the coagulation cascade since it catalyzes the formation of thrombin from prothrombin, which is common to both the intrinsic and extrinsic activation pathways. The formation of thrombin catalyzed by fX_a occurs following the assembly of a catalytic complex (prothrombinase) composed of fX_a, the nonenzymatic cofactor V_a, and prothrombin assembled on an appropriate phospholipid surface (i.e., activated platelets) in the presence of Ca^{2+}. The unique position of fX_a at the point of convergence of the two cascade pathways illustrates its importance in blood coagulation and makes it an intriguing target for inhibition in the possible treatment of occlusive vascular disease.

Tick anticoagulant peptide (TAP) is a potent inhibitor of fX_a isolated from the tick *Ornithodoros moubata* (Waxman et al., 1990). TAP is a protein composed of 60 amino acid residues, including 6 cysteines. Although TAP exhibits limited homology to the Kunitz class of inhibitor, the spacing of the six cysteine residues is not strictly maintained, and the p*I* (4.5) is much more acidic than the generally basic Kunitz inhibitors (Laskowski & Kato, 1980). Most serine proteinase inhibitors share a common mechanistic pathway consisting of binding of the proteinase through its active site to the reactive site of the inhibitor followed by the slow cleavage of a peptide bond within the inhibitor's reactive site, yielding a covalently modified enzyme–inhibitor complex (Laskowski & Kato, 1980). This type of reaction occurs with inhibitors in the Kunitz class, the serpins and antistasin, a protein recently isolated from the leech *Haementeria officinalis*, which inhibits both fX_a and trypsin (Dunwiddie et al., 1989). This mechanism requires that the reactive site of the inhibitor resembles the corresponding region of the substrate recognized by the enzyme. Therefore, inhibitors with Lys and Arg in the P_1 position would be recognized by both trypsin and trypsin-like enzymes. In contrast, TAP exhibits a very high degree of specificity, inhibiting only fX_a.

Hirudin, a small peptide (65 amino acids) containing 6 cysteine residues, is also a highly specific proteinase inhibitor whose target enzyme is thrombin. Hirudin binds thrombin with high affinity (K_i = 20 fM; Stone & Hofsteenge, 1986) and has been shown to have a unique mechanism involving two separate binding sites, one of which is at or near the active site of thrombin (Stone & Hofsteenge, 1986). However, neither binding site is cleaved by thrombin nor does it mimic the substrate as do other serine proteinase inhibitors, including the Kunitz inhibitors. Considering the similarities between TAP and hirudin, the possibility exists that TAP may utilize a mechanism similar to that of hirudin.

In order to determine the mechanism of inhibition of fX_a by TAP, it is essential to examine the kinetics of this interaction. Due to the limited quantities of the native inhibitor isolated from ticks, we utilized a recombinant version of TAP (rTAP) expressed in the yeast *Saccharomyces cerevisiae* (Neeper et al., 1990) in these studies. We report here the results of these studies which describe the kinetic inhibition of fX_a by rTAP and discuss the possible mechanistic implications of these results and their relationship to the mechanisms of other, well-characterized proteinase inhibitors.

Experimental Procedures

Materials. Human factor X_a (hfX_a) was purchased from Enzyme Research Laboratories, Inc., and was determined to be homogeneous by reducing and nonreducing SDS–PAGE. The enzyme concentration was determined by active-site titration using 4-methylumbelliferyl *p*-guanidinobenzoate hydrochloride (MUGB) (Jameson et al., 1973). The substrate, Spectrozyme X_a (methoxycarbonyl-D-cyclohexylglycylglycylarginine-*p*-nitroanilide acetate), was purchased from

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[1] Abbreviations: DFP, diisopropyl fluorophosphate; DIP, diisopropylphosphoryl; fX_a, factor X_a; hfX_a, human factor X_a; LACI, lipoprotein-associated coagulation inhibitor; MUGB, 4-methylumbelliferyl *p*-guanidinobenzoate hydrochloride; TAP, tick anticoagulant peptide; TBSA, Tris-buffered saline plus 0.1% bovine serum albumin; Tris, tris(hydroxymethyl)aminomethane; rTAP, recombinant tick anticoagulant peptide.

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American Diagnostica, Inc. Recombinant tick anticoagulant peptide (rTAP) was produced as a secreted protein using the yeast *Saccharomyces cerevisiae* which is described in a separate publication (Neeper et al., 1990). rTAP was quantitated by using amino acid analysis. All other reagents were obtained from commercial sources and were of analytical grade.

Enzyme Assays. Enzyme activity was determined by monitoring the increase in absorbance at 405 nm caused by the release of *p*-nitroaniline when the substrate is hydrolyzed (E_{405} = 9920 M^{-1} cm^{-1}; Lottenberg & Jackson, 1982). The reactions were performed in 96-well microtiter plates (Dynatech) and followed with time using a V-max or Thermo-max kinetic microplate reader (Molecular Devices) at room temperature. Unless otherwise stated, all assays were performed in a total volume of 220 μL in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 0.1% bovine serum albumin (TBSA) with a final concentration of 500 pM fX_a. Under these conditions, the K_m for substrate hydrolysis was determined to be 65 ± 5 μM by nonlinear regression analysis, assuming Michaelis–Menten kinetics. The concentrations of rTAP and substrate were varied as described in the text. In all cases, fX_a and rTAP were diluted into TBSA just prior to use and maintained on ice. Spectrozyme X_a was dissolved in double-distilled water to 6.67 × 10^{-3} M before being diluted into TBSA. The substrate was maintained at room temperature in the dark.

In order to determine the inhibition of fX_a by rTAP, solutions containing fX_a plus increasing concentrations of rTAP were preincubated for 30 min at room temperature, and the initial velocities of residual enzyme activity were monitored for 5 min after the addition of substrate. Time course assays were started by the addition of fX_a to solutions containing substrate and rTAP, and were monitored for 30 min.

Determination of k_{off}. In order to determine the dissociation rate constant directly, fX_a (10 nM) and rTAP (20 nM) were incubated for 30 min, and the enzyme–inhibitor complex was isolated by loading 100 μL of the solution onto a 2-mL spin column (Boehringer Mannheim) containing Sephadex G-50 (Pharmacia Fine Chemicals). The column had been preequilibrated with TBS and precentrifuged (Jouan CR 4.11) at 1800*g* for 2 min. The loaded column was centrifuged (Jouan MR 15) at 4700*g* for 10 min. The eluate containing the fX_a–rTAP complex was diluted 50-fold into TBSA. The return of the activity of fX_a (representing the amount of free fX_a present in solution) was monitored by removing aliquots of this solution at various times after dilution and measuring the initial rate velocities ([substrate] = 300 μM). The reaction was followed over a 6-h period.

Direct Binding Experiments. Preparation of DFP-inactivated human fX_a was done according to a method described by Broze and Miletich (1987). The resulting DIP–fX_a was 99% inactivated using the chromogenic fX_a assay. ^{35}S-rTAP was purified to homogeneity as described (Neeper et al., 1990) and had a specific radioactivity of 162000 cpm/μg. ^{35}S-rTAP was incubated with human fX_a, DIP–fX_a, or zymogen factor X for 1 h in TBSA at 25 °C prior to separation on a 1.0 × 45 cm Sephadex G-75 Superfine column equilibrated with the same buffer at 0.3 mL/min. Radioactivity in the resulting fractions (1 mL) was determined by liquid scintillation counting while fX_a activity was determined by using the chromogenic assay described above.

RESULTS

Determination of the Dissociation Constant. To characterize the nature of the interaction between rTAP and fX_a, we first attempted to obtain a value for the dissociation constant of the fX_a–rTAP complex. We also investigated whether the inhibition of fX_a by rTAP demonstrates slow-binding kinetics as seen with many polypeptide inhibitors of serine proteinases.

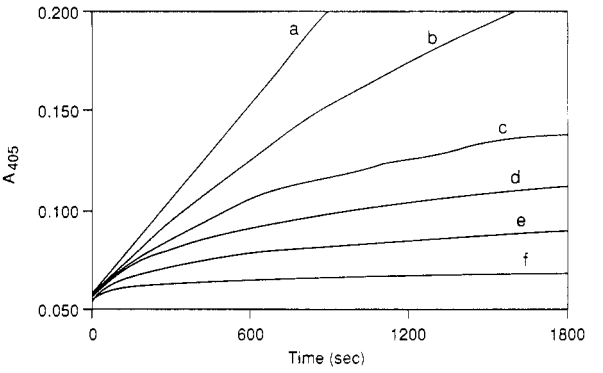


FIGURE 1: Progress curves illustrating the slow inhibition of fX_a by rTAP. The tracings were recorded over 30 min for reactions initiated by the addition of hfX_a (0.50 nM) to 200 μM Spectrozyme X_a and increasing concentrations of rTAP in TBSA. rTAP concentrations: (a) 0 nM; (b) 1 nM; (c) 3 nM; (d) 5 nM; (e) 8 nM; (f) 20 nM.

Preliminary experiments which involved preincubating rTAP with enzyme at room temperature for various times prior to the addition of substrate suggested that a steady state between enzyme and inhibitor was achieved after 15 min (data not shown). The slow-binding nature of the fX_a–rTAP interaction was confirmed when reactions initiated by the addition of hfX_a to substrate containing increasing concentrations of rTAP produced progress curves similar to those described by Morrison (1982) for slow-binding inhibitors (Figure 1). The steady-state velocities measured at the end of the time course assays agree well with the initial steady-state velocities obtained when rTAP is preincubated with fX_a prior to the addition of substrate, indicating that a true, inhibited steady state is reached during the 30-min assay. Since substrate depletion was insignificant over the course of the assays (less than 13% for the lowest concentration of substrate tested), the steady-state velocities determined over this time course were considered valid.

Besides exhibiting slow-binding kinetics, rTAP exhibits significant inhibition of fX_a at concentrations similar to that of the enzyme, suggesting that rTAP is also a tight-binding inhibitor (Williams & Morrison, 1979). In addition, the stoichiometric inhibition of fX_a with rTAP plus active-site titration experiments performed in the absence and presence of a known concentration of rTAP indicates that the inhibitor is fully active toward fX_a (Jordan, unpublished observations).

Conventional Michaelis–Menten kinetics are not applicable for the analysis of tight-binding inhibitors (Morrison, 1969; Cha, 1975a), as they do not account for inhibitor depletion which occurs during the inhibition reaction. Therefore, Morrison's equation for tight-binding inhibition (Morrison, 1969) was used to obtain a dissociation constant for rTAP (K_i):

$$V_s/V_0 = \{([E_t] - [I_t] - K_i^*) + [([I_t] + K_i^* - [E_t])^2 + 4K_i^*[E_t]]^{1/2}\}/2[E_t] \quad (1)$$

where K_i^* is the apparent dissociation constant for the enzyme–inhibitor complex, V_s is the inhibited steady-state velocity, V_0 is the uninhibited (control) velocity, $[I_t]$ is total inhibitor conentration, and $[E_t]$ is the total fX_a concentration. In these experiments, the enzyme (0.5 nM) and inhibitor (0.1–20 nM) were preincubated for 30 min to allow equilibration of the enzyme–inhibitor complex. Reactions were initiated by the addition of substrate (200 μM). Since rTAP and fX_a are already at equilibrium and the dissociation rate

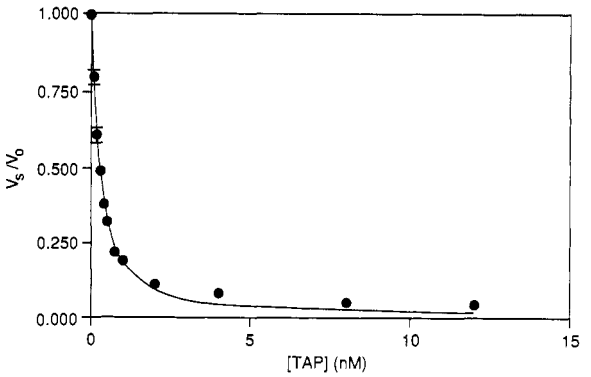


FIGURE 2: Determination of the intrinsic K_i^* from steady-state velocities. The reactions were initiated by the addition of Spectrozyme X_a (200 μM) to solutions of hfX_a (0.50 nM) and increasing concentrations of rTAP (0–12 nM) that had been preequilibrated with fX_a for 30 min (n = 15). The initial velocities were measured for 5 min. The solid line represents the best nonlinear fit of the data to eq 1, yielding K_i^* = 0.18 ± 0.02 nM.

of the fX_a–rTAP complex is slow (see below), the initial rate of the reaction corresponds to the inhibited steady-state velocity (V_s). When the resulting initial rate data were fit by nonlinear regression to eq 1, K_i^* was calculated as 0.18 ± 0.02 nM (Figure 2). Similar results were obtained at all substrate concentrations tested (70–500 μM) (data not shown). This intrinsic dissociation constant is independent of the nature of the inhibition (competitive vs noncompetitive) since the enzyme and inhibitor were already at equilibrium prior to the addition of substrate.

Determination of Inhibition Mechanism. In order to determine the type of inhibition exhibited by rTAP, the progress curves obtained in Figure 1 were analyzed by using the rate equations of Williams and Morrison (1979) and Cha (1975a). The integrated first-order rate equation (eq 2) describes the

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

slow establishment of equilibrium between enzyme and inhibitor where P is the measured absorbance defined as a function of the initial (V_0) and final (V_s) steady-state velocities and the apparent first-order rate constant, k_{obs}, which describes the equilibration from the initial to the final steady state. The progress curves in Figure 1 were fit by using nonlinear regression to this equation to obtain k_{obs} at varying inhibitor concentrations. The values obtained for k_{obs} and V_s were similar to those obtained by fitting the data to a more complicated equation (Cha, 1976; Williams et al., 1979) which takes into account the depletion of enzyme and inhibitor during the reaction (data not shown).

The steady-state velocities (V_s) obtained at different inhibitor concentrations (0.5–20 nM) by fitting the time course data to eq 2 were fit by nonlinear regression to eq 1 for tight-binding inhibitors to obtain values for K_i^*. If the inhibition is noncompetitive, then K_i^* will be equal to K_i, and independent of the concentration of substrate used in the assay. However, if the inhibition is competitive, K_i^* will be dependent on substrate concentration, which can be described by the expression $K_i^* = K_i(1 + [S]/K_m)$. When this method was used, values for K_i^* were calculated at various substrate concentrations (70–400 μM). The results of this analysis show a linear relationship between K_i^* and substrate concentration (Figure 3), which indicates that the inhibition of fX_a by rTAP is competitive. A plot of K_i^* versus substrate concentration further predicts an intercept of K_i. By use of linear regression analysis of the results in Figure 3, an intercept of 0.142 ± 0.03 nM was calculated (r = 0.9818). This value is in reasonable

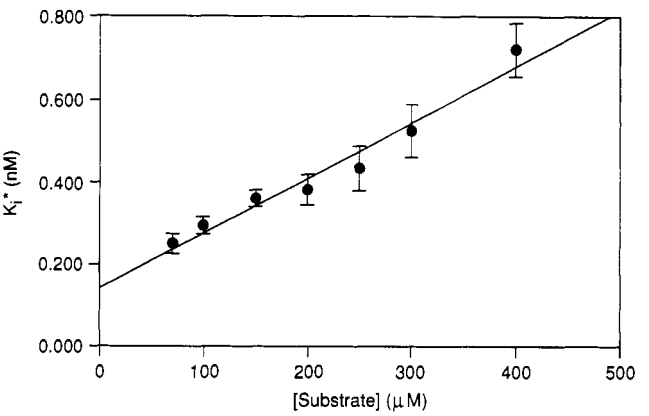
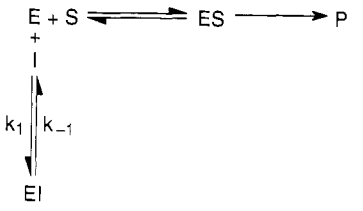
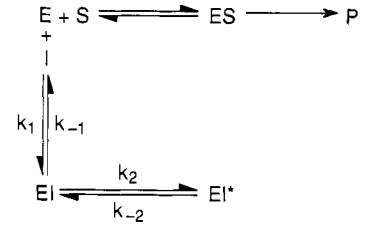


FIGURE 3: Relationship of the apparent dissociation constant, K_i^*, to substrate concentration. Values for K_i^* were calculated as described in the text ([hfX_a] = 0.5 nM; [rTAP] = 0–20 nM; [Spectrozyme X_a] = 70–400 μM. The plot represents a summary of five experiments at each substrate concentration. Linear regression analysis of the data results in the solid line shown (r = 0.9818).

Scheme I



Scheme II



agreement with the intrinsic K_i^* calculated above.

Further evidence to support the conclusion that rTAP is a competitive inhibitor of fX_a was sought by using qualitative binding experiments to determine whether rTAP binds to the active site of fX_a. Panel A of Figure 4 shows the separate elution profiles of active fX_a and ^{35}S-rTAP. As illustrated in panel B (Figure 4), when ^{35}S-rTAP is preincubated with fX_a to equilibrium, the bound ^{35}S-rTAP exhibits a similar elution profile as that expected for a fX_a–rTAP complex. However, ^{35}S-rTAP which has been incubated with a large excess of DFP-inactivated fX_a elutes at the same position as that of rTAP alone, indicating that rTAP does not bind with high affinity to the modified fX_a. Similarly, ^{35}S-rTAP does not bind to zymogen factor X (panel C, Figure 4). These results indicate that rTAP requires the active-site serine for binding to fX_a since this residue is covalently modified by DFP (Fenton et al., 1981) and is inaccessible in the zymogen fX. Furthermore, these data suggest that rTAP does not bind with high affinity to secondary sites on fX_a.

Determination of Rate Constants for Formation and Dissociation of EI Complexes. Slow-binding, competitive inhibition can be described by more than one mechanism, and two possible models have been postulated (Morrison, 1982). Scheme I predicts the slow formation of a single EI complex, while Scheme II postulates the rapid formation of an EI complex which then slowly isomerizes to a more stable complex (EI*). These two schemes are described by different rate

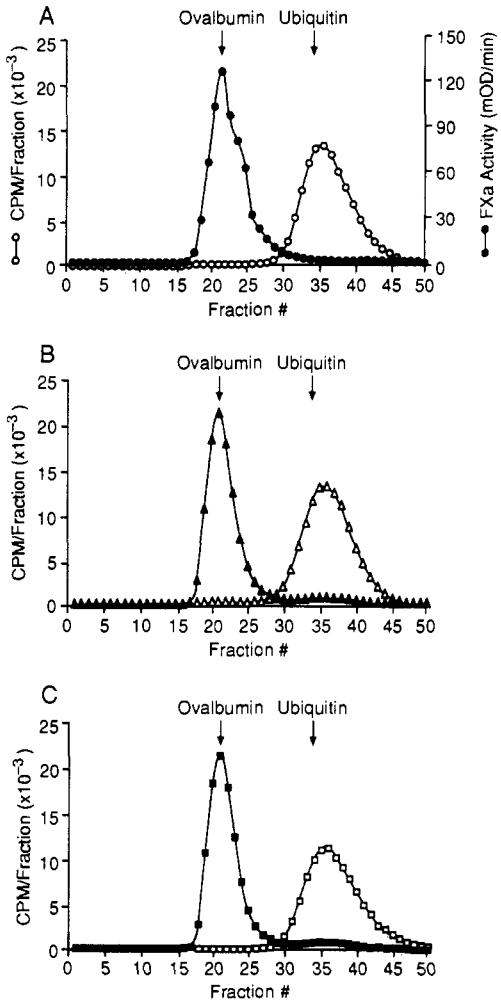


FIGURE 4: Elution profiles of ^{35}S-rTAP incubated with fX_a, DIP-fX_a, or zymogen fX. Samples in 100 μL were prepared as described under Experimental Procedures, loaded onto a G-75 Superfine column, and eluted with TBSA at 0.3 mL/min. Radioactivity or enzymatic activity was determined for each 1-mL fraction as described under Experimental Procedures. The elution of ovalbumin (M_r = 43 000) and ubiquitin (M_r = 8565) is shown as standards. Panel A: fX_a alone (0.91 nmol) (●); ^{35}S-rTAP alone (0.61 nmol) (○). Panel B: ^{35}S-rTAP (0.61 nmol) plus fX_a (1.82 nmol) (▲); ^{35}S-rTAP (0.61 nmol) plus DIP-fX_a (9 nmol) (△). Panel C: ^{35}S-rTAP (0.61 nmol) plus fX_a (1.82 nmol) (■); ^{35}S-rTAP (0.61 nmol) plus zymogen fX (1.8 nmol) (□).

equations (Williams & Morrison, 1979; Morrison, 1982). In Scheme I, there is a linear relationship between inhibitor concentration and the apparent first-order rate constant, k_{obs}, while Scheme II predicts a hyperbolic relationship (eqs 3 and 4, respectively) where $K_{i,app} = K_i(1 + [S]/K_m)$ and $K_i = k_{-1}/k_1$.

$$k_{obs} = k_{-1} + k_1[I]/(1 + [S]/K_m) \quad (3)$$

$$k_{obs} = k_{-2} + k_2[I]/([I] + K_{i,app}) \quad (4)$$

The rate constants, k_{obs}, determined by fitting the progress curves (Figure 1) to eq 2 were fit by nonlinear regression to eq 3 and 4. The data fit to eq 3 (Scheme I) result in a linear relationship between k_{obs} and inhibitor (r = 0.9965) (Figure 5). The corresponding values for k_1 and k_{-1} were calculated as (2.85 ± 0.07) × 10^6 M^{-1} s^{-1} and (0.554 ± 0.17) × 10^{-3} s^{-1}, respectively. From these values, the estimated dissociation constant was calculated to be 0.19 nM. This is in excellent agreement with the previously determined intrinsic value for K_i* (see above). Alternatively, the data were fit to eq 4 (Scheme II), and values were calculated for k_2, k_{-2}, and $K_{i,app}$ [207.3 ± 282.3 s^{-1}, (0.718 ± 0.319) × 10^{-3} s^{-1}, and (3.098 ± 4.225) × 10^{-4} M, respectively]. These values were then used to calculate the estimated dissociation constant (eq 5), yielding

$$K_{i,overall,app} = K_{i,app}[k_{-2}/(k_2 + k_{-2})] \quad (5)$$

a value of 1.07 nM. This value does not agree with the previously determined intrinsic K_i*. In addition, the large errors in the calculated rate constants for Scheme II, the good correlation of the calculated dissociation constant with the calculated intrinsic K_i* when Scheme I is assumed, and the fact that the plot of k_{obs} vs [rTAP] exhibits no evidence for any deviation from linearity all suggest that if a preliminary EI complex is formed, as predicted in Scheme II, it is too weak to be detected with the experimental procedures used.

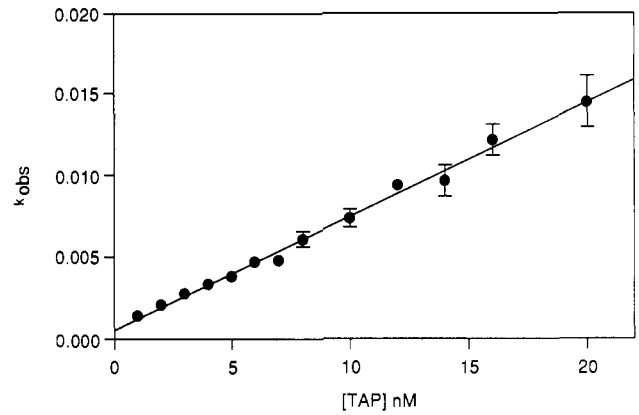


FIGURE 5: Relationship between the apparent first-order rate constant, k_{obs}, and the concentration of rTAP. Progress curves were generated with 200 μM Spectrozyme X_a, 0.5 nM hfX_a, and 0–20 nM rTAP. The apparent first-order rate constant was calculated by using a nonlinear regression fit of the data to eq 2. The solid line represents the best fit of the data to eq 3 (r = 0.9965), yielding k_1 = (2.85 ± 0.07) × 10^6 M^{-1} s^{-1}, k_{-1} = (0.554 ± 0.178) × 10^{-3} s^{-1}, and K_i* = 0.19 nM where K_m = 65 μM. (Summary of eight experiments.)

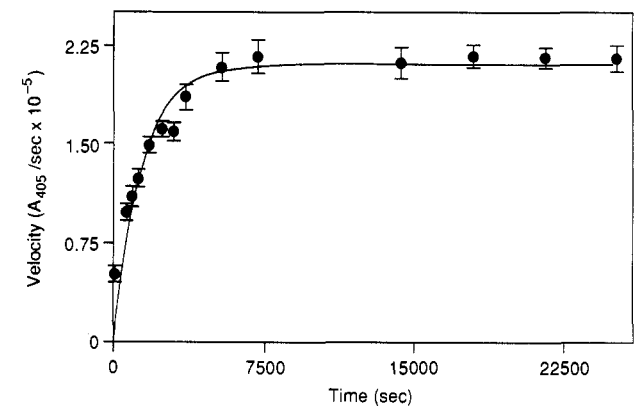


FIGURE 6: First-order rate plot for the dissociation of hfX_a and rTAP. The reaction was performed as described under Experimental Procedures. The initial velocities were measured for 5 min. The solid line represents a nonlinear regression fit of the data to eq 6, yielding k_{-1} = (0.725 ± 0.087) × 10^{-3} s^{-1} (summary of 10 experiments).

The calculated dissociation rate constant (k_{-1} or k_{off}), using eq 3, has a large error that is probably due to its very small value. A second direct method, as described under Experimental Procedures, was therefore used to determine its value. Figure 6 shows a first-order rate plot of the recovery of fX_a activity after preincubation with rTAP. A nonlinear fit of the data to eq 6 is given by the solid line which yields a value of

$$V = a(1 - e^{-kt}) \quad (6)$$

(0.725 ± 0.087) × 10^{-3} s^{-1}. In eq 6, V represents the observed velocity of the reaction as a function of time, t, and k is the calculated dissociation rate constant. The calculated value

is probably a slight overestimate of the true dissociation rate constant since eq 6 does not take into account the reassociation of rTAP with fX_a. Even without correcting for this back-reaction, the value is in good agreement with the value determined above using Scheme I. Since both Schemes I and II assume competitive inhibition, the agreement between the calculated value for k_{off} and that obtained by direct measurement indicates that the inhibition of fX_a by rTAP is indeed competitive. This experiment also clearly illustrates the reversibility of the inhibition reaction.

Discussion

To gain insight into the inhibition mechanism of fX_a by rTAP, a detailed steady-state kinetic analysis was performed on the interaction of the enzyme with the inhibitor. rTAP has been shown to be a slow, tight-binding competitive inhibitor of fX_a with a calculated dissociation constant of 1.8×10^{-10} M, which is about 3-fold lower than that reported by Waxman et al. (1990) for native, tick-derived TAP. It is not known if this discrepancy reflects a real difference between the recombinant and the native proteins or if it is simply due to difficulty in quantifying the small amounts of native inhibitor available in the initial study. Studies have now been initiated to try to resolve this issue. The reversibility of the inhibition of fX_a is illustrated by the return of fX_a activity in experiments used to directly determine a value for the dissociation rate constant of the EI complex. Furthermore, qualitative binding studies which show that rTAP binds only to fX_a and not DIP–fX_a or zymogen factor X indicate that the peptide requires the active site for high-affinity binding to fX_a.

Our results also indicate that the inhibition of fX_a by rTAP, at the limiting concentrations used in this study, conforms with Scheme I (Morrison, 1982). Therefore, if an initial EI complex is formed prior to rearrangement to a more stable EI* complex, as in Scheme II, it is too weak to be observed under the current experimental conditions. Further studies are currently under way to elucidate whether a preliminary complex is formed. Recently, other examples of slow, tight-binding inhibitors which can be described by Scheme I have been reported (Bull et al., 1985; Stone et al., 1987b; Schmidt et al., 1989).

The small size of TAP (60 amino acids) and its amino acid sequence, including 6 cysteine residues, suggest that it is related to the Kunitz class of serine proteinase inhibitors (Laskowski & Kato, 1980; Waxman et al., 1990). However, certain differences exist between the Kunitz inhibitors and TAP including the lack of a Kunitz-type reactive site in TAP and a much more acidic p*I* than is typical for the family of basic Kunitz inhibitors (Waxman et al., 1990). Furthermore, although having some specificity for their target enzyme, all Kunitz inhibitors also inhibit trypsin to some extent, whereas TAP does not. Interestingly, bovine pancreatic trypsin inhibitor, which inhibits trypsin with a K_i of 10 pM, has no effect on fX_a (Auerswald et al., 1988).

Despite the above differences, a comparison of the kinetic characteristics of rTAP with known Kunitz inhibitors reveals certain similarities. For instance, the dissociation constants of three such inhibitors for their preferred enzyme targets [trypstatin (1.2×10^{-10} M; Kido et al., 1988), lipoprotein-associated coagulation inhibitor (LACI) (6.6×10^{-10} M; Broze et al., 1987), and an inhibitor isolated from *Drosophila funebris* (3.7×10^{-9} M; Schmidt et al., 1989)] are all similar to that reported here for rTAP. Furthermore, all three of these Kunitz inhibitors are tight-binding inhibitors, and both LACI and the *Drosophila* peptide exhibit slow-binding inhibition kinetics as well. Although LACI and the *Drosophila* peptide are competitive inhibitors, the inhibition of fX_a by LACI conforms with Scheme II (Broze et al., 1987) while the *Drosophila* inhibitor is similar to rTAP in that its inhibition of acrosin conforms with Scheme I (Schmidt et al., 1989).

Another proteinase inhibitor, antistasin, which is isolated from the leech *Haementeria officinalis*, was found to inhibit both fX_a and trypsin (Nutt et al., 1988; Dunwiddie et al., 1989). Like rTAP, antistasin has been characterized as a mostly competitive, slow, tight-binding inhibitor with a dissociation constant for fX_a between 0.31 and 0.62 nM (Dunwiddie et al., 1989). There are, however, some important differences between rTAP and antistasin. First, when small chromogenic substrates are used, Ca^{2+} affects the inhibition of fX_a by antistasin but not by TAP (G. Vlasuk and D. Smith, unpublished observations). Furthermore, while antistasin resembles many other serine proteinase inhibitors (including Kunitz inhibitors) in that it is specifically cleaved by its target enzyme at a specific peptide bond (Dunwiddie et al., 1989; Laskowski & Kato, 1980), there is no evidence for such cleavage occurring with rTAP, even after 24 h of incubation with fX_a at 37 °C (G. Vlasuk, unpublished observations). Third, antistasin inhibition conforms with Scheme II in contrast with rTAP, whose inhibition profile more closely resembles that of Scheme I. It has been postulated by Dunwiddie et al. (1989) that the second, slow step of Scheme II may be a transitional state generated during the process of the cleavage reaction. Since rTAP is not cleaved, it is not surprising that inhibition by rTAP conforms with Scheme I rather than Scheme II.

The proteinase inhibitor hirudin, which is highly specific for thrombin, exhibits tight-binding kinetics (K_i = 20 fM; Stone & Hofsteenge, 1986), is similar to TAP in size (65 amino acids) and number of cysteine residues (6 each), and is not cleaved by its target proteinase, all of which suggest that the inhibition mechanism of rTAP may resemble that of hirudin. In addition, the dissociation constants of both inhibitors are dependent on substrate concentration, which indicates competitive inhibition. However, in the case of hirudin, the relationship is parabolic rather than linear, which suggests that hirudin interacts with more than one site. The first site is most likely at or near the active site of the enzyme, while the second site may be elsewhere on the thrombin molecule or the substrate itself (Stone & Hofsteenge, 1986). Furthermore, hirudin is able to bind to thrombin even when the active site of thrombin is blocked, although 10^3 times less tightly (Stone et al., 1987a), while we show here that rTAP is not able to bind with high affinity to fX_a in which the active site has been modified by DFP, which indicates that in contrast with hirudin, rTAP lacks any secondary high-affinity binding sites.

That the two inhibitors are kinetically dissimilar is further illustrated by the effect of ionic strength on their dissociation constants. Not only is there a marked increase in the apparent K_i of the hirudin–thrombin complex with increasing ionic strength (due solely to a decrease in $k_{1'}$), but also slow-binding kinetics are only observed at an ionic strength above 0.2 (Stone & Hofsteenge, 1986). In contrast, the dissociation constant of rTAP with fX_a is not appreciably altered over a wide range of ionic strength (Table I), which indicates that the initial binding of rTAP to fX_a is not dependent primarily on ionic interactions as has been proposed for the binding of hirudin to thrombin (Stone & Hofsteenge, 1986). However, because of the similarities between the two inhibitors, the absence of evidence for a second binding site for rTAP does not eliminate the possibility that the interaction of rTAP with the active site of fX_a may be similar to that of hirudin with the active site of thrombin. It is of particular interest that the dissociation

Table I: Effect of Ionic Strength on Intrinsic K_i^*

[NaCl][a] (mM)	K_i^{*b} (nM)
75	0.173
150	0.180
750	0.143

[a] The reaction buffer also consisted of 50 mM Tris-HCl, pH 7.5, containing 0.1% bovine serum albumin. [b] K_i^* was calculated as described in the text.

rate constants of rTAP (0.554×10^{-3} s^{-1}) and hirudin (0.56×10^{-3} s^{-1}; Stone & Hofsteenge, 1986) are similar. This suggests that once formed, the fX_a–rTAP complex is as stable toward dissociation as the thrombin–hirudin complex.

The structural and kinetic evidence accumulating regarding tick anticoagulant peptide suggests that it is a new type of proteinase inhibitor that is highly specific for its target enzyme, fX_a. It will be of interest to determine whether the kinetic profile described in this paper is similar when TAP is reacted with fX_a that is bound to factor V_a, phospholipid, Ca^{2+}, and its physiological substrate prothrombin. Also, further studies to determine which part of the TAP molecule binds to the active site of fX_a will provide insight into the mechanism of this novel proteinase inhibitor.

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