

Draculin, the anticoagulant factor in vampire bat saliva, is a tight-binding, noncompetitive inhibitor of activated factor X

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Draculin, the anticoagulant factor in vampire bat saliva, is a tight-binding, noncompetitive inhibitor of activated factor X

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Abstract

The kinetic mechanism of action of Draculin on activated Factor X (FXa) is established. Draculin inhibits activated Factor X within seconds of incubation at near equimolar concentration (2–6 times on molar basis). Fitting the data to the equation for a tight-binding inhibitor gives a value for $K_i(K_d) = 14.8 \pm 1.5$ nM. The formation of the Draculin–FXa complex can be explained by a two-step mechanism, where for the first, reversible step, $k_{on} = 1.117 (\pm 0.169, \text{S.E.M.}) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and $k_{off} = 15.388 (\pm 1.672) \times 10^{-3} \text{ s}^{-1}$, while for the second, irreversible step, which is concentration-independent, $k_2 = 0.072 \text{ s}^{-1}$. K_d obtained from $k_{off}/k_{on} = 13.76$ nM. Lineweaver–Burk plot shows a noncompetitive behavior. This noncompetitive mode of inhibition of Draculin is supported by the observation that Draculin, at concentrations giving complete inhibition, does not impair binding of *p*-aminobenzamidine to FXa. Moreover, under the same conditions, Draculin induces <14% decrease of the fluorescence intensity of the *p*-aminobenzamidine–FXa complex. We conclude that Draculin is a noncompetitive, tight-binding inhibitor of FXa, a characteristic so far unique amongst natural FXa inhibitors. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: FXa Inhibition; Draculin; Tight-binding inhibitor; Natural anticoagulant; (Vampire bat)

1. Introduction

Activation of Factor X is a common point between the intrinsic and extrinsic pathway of blood coagulation [1]. Activated factor X (FXa) is the sole enzyme that catalyzes the conversion of prothrombin into thrombin, the key enzyme in the coagulation cascade; therefore inhibitors of this step are of considerable mechanistic and pharmacological interest. In addition to the two known physiological inhibitors of this serine protease, antithrombin III (AT-III) and the tissue factor pathway inhibitor (TFPI), several low molecular mass naturally occur-

Abbreviations: FXa, activated Factor X; FIXa, activated Factor IX; S.E.M., standard error median; pAB, *p*-aminobenzamidine; BA, benzamidine; AT-III, Antithrombin III; TFPI, tissue factor pathway inhibitor; TAP, tick anticoagulant peptide; rTAP, recombinant TAP; AcAP, *Ancylostoma caninum* anticoagulant peptide; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HRP, horseradish peroxidase; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant

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ring polypeptides inhibitors have been described, such as Antistasin, a 119-residue protein isolated from the mexican leech *Haementeria officinalis* [2,3]; the tick anticoagulant peptide (TAP) [4], a 60-amino acid protein derived from the tick *Ornithodoros moubata*; Ecotin, a periplasmic protein found in *Escherichia coli* [5–7]; and the *Ancylostoma caninum* anticoagulant peptide (AcAP) [8]. These peptides behave as reversible, slow tight-binding inhibitors of FXa, where Antistasin and Ecotin are slowly cleaved by FXa, while TAP is not affected by the protease. Recently, we described a new natural FXa inhibitor, isolated from the saliva of the vampire bat *Desmodus rotundus*, which was named Draculin. Draculin is a 88.5 kDa glycoprotein which selectively inhibits both FXa and activated factor IX (FIXa) [9]. Furthermore, the anticoagulant activity of Draculin is highly dependent on the proper glycosylation of the polypeptide backbone [10]. The results described in this paper indicate that, in contrast to other natural FXa inhibitors, Draculin behaves as a noncompetitive, tight-binding inhibitor of FXa.

2. Materials and methods

2.1. Materials and reagents

Hydroxyapatite (Biogel-HTP), acrylamide and silver-staining reagents were purchased from Bio-Rad Laboratories (Richmond, CA, USA). Benzamidine and *p*-aminobenzamidine dihydrochloride were from Sigma (MO, USA). Coagulation factors were purified by established procedures by Dr. Rob Wagenvoort (Department of Biochemistry, University of Limburg, The Netherlands). FXa concentration was measured by titration with *p*-*N*-*p*-guanidobenzoate [11]. The activity of FXa was assayed using the chromogenic substrates S-2222 ($K_m = 200 \mu\text{M}$, Chromogenix, Sweden), S-2337 ($K_m = 200 \mu\text{M}$, Kabi Diagnostica, Sweden) and $\text{CH}_3\text{OCO-D-CHG-Gly-Arg-pNA}$ ($K_m = 104 \mu\text{M}$, Pentapharm). Freund's complete adjuvant (FCA) was from Calbiochem (CA, USA) and Freund's incomplete adjuvant (FIA) was from Gibco (OH, USA). EGGstract purification system and Anti-chicken IgY HRP conjugate were supplied by Promega (WI, USA). ECL

Western blotting detection reagents were purchased from Amersham Life Science (UK). All other reagents used were of the highest quality commercially available. For graphics and mathematical approaches, Origin 5.0 Software was used (Microcal Software, MA, USA).

2.2. Animals

Vampire bats (*Desmodus rotundus*) were regularly captured from wild colonies in the region of Choroni (State of Aragua, Venezuela). The bats were kept in captivity, in individual cages of the metabolic type (Acme Metal Products, Chicago, IL, USA), under controlled light and temperature (25°C). The animals were maintained on bovine blood anticoagulated with 3.8% sodium citrate at a ratio of 1:9. Food was given every 24 h and water was given ad libitum [12].

2.3. Saliva collection

As shown previously [10], the biological activity of Draculin is highly dependent on the appropriate glycosylation of the native protein and seriously affected by the salivation pattern of the animals. Therefore, for the purpose of the present study, saliva collection was done as follows: eight to ten animals were salivated, as previously described [10], at 5-day intervals. Each sample was assayed for protein concentration and FXa inhibitory activity, and kept at -20°C until use. Similar batches were pooled and Draculin purified as already described [10]. Under these conditions, the purified protein obtained had an inhibitory activity of 4.92 nmol FXa inhibited per mg of Draculin.

2.4. Factor Xa activity

Factor Xa activity was assayed on specific chromogenic substrates. For the microplate assay of FXa activity, 50 μl of each of the hydroxyapatite column-eluted fractions was placed in a well with 100 μl of buffer A (0.05 M Tris-HCl, 0.1 M NaCl (pH 7.35) with 0.05% egg albumin), 3 nM FXa and 20 μl of 4 mM S-2222, in a final volume of 180 μl . After 15 min incubation at 37°C the optical density at 405 nm was recorded in a microplate reader

(EL312e Bio-Kinetics reader microplate, Bio-Tek Instruments). Continuous assay of FXa activity was used for quantifying and kinetics studies of the anti-Xa activity of Draculin. Briefly, 10–13 nM FXa was incubated with Draculin for 1 min at 37°C in a cuvette containing buffer A. The cuvette was placed in a home-made fixed dual-wavelength photometer (Biochemistry and Instrumental Dept., Univ. of Limburg, The Netherlands) for the continuous recording of the reaction at 405 nm. The reaction was started with addition of 35 μ l of S-2337 (final concentration = 400 μ M). The final volume of the reaction mix was 500 μ l. For kinetic studies, CH₃OCO-D-CHG-Gly-Arg-pNA, a low- K_m substrate, was used and FXa was in the range of 1–4 nM. The reaction conditions were as described above.

2.5. *p*-Aminobenzamide binding to FXa

Use of *p*-Aminobenzamide (pAB) as a fluorescent probe for the active site of serine proteases and its displacement by active site specific ligands (benzamide, AT III) has been previously reported [13,14]. FXa (1 μ M) and pAB (75 μ M) were incubated in a microcuvette (2 mm wide, 10 mm path) with buffer A (final volume = 500 μ l). The fluorescence intensity was measured in a spectrofluorophotometer (RF-1501, Shimadzu) with excitation at 336 nm and emission at 376 nm. Draculin (7 μ M) was added as described in Fig. 6. Benzamide (1.13 mM) was used as a positive control for pAB displacement.

2.6. HPLC of the Draculin–FXa complex

Draculin and FXa at a ratio of 1:10 were incubated for 10 min at room temperature. A 25- μ l sample was loaded on a molecular exclusion column (Protein Pak 300sw, Waters, Millipore, USA). Equilibration of the column and elution of the sample was done with 250 mM phosphate, pH 6.8. Draculin at the same concentration was run as control. Detection was monitored at 280 nm.

2.7. Effect of factor Xa on Draculin

In order to determine if the interaction of Draculin with FXa results in cleavage of the inhibitor, 200 nM FXa and 400 nM Draculin were incubated at room temperature, and aliquots of the reaction mixture were removed at various time points and subjected to Western blotting analysis using anti-Draculin antibodies from egg yolks, obtained as previously described [10]. Detection was done using a chemiluminescent method (Amersham Life Science). Factor Xa and Draculin incubated alone under the same conditions were used as controls.

2.8. Other methods

Protein determination was done by the Coomassie blue method [15]. SDS–PAGE was done as described [16]. Gels were stained with Coomassie blue or silver staining. For Western blotting analysis, appropriate methods were used [17,18].

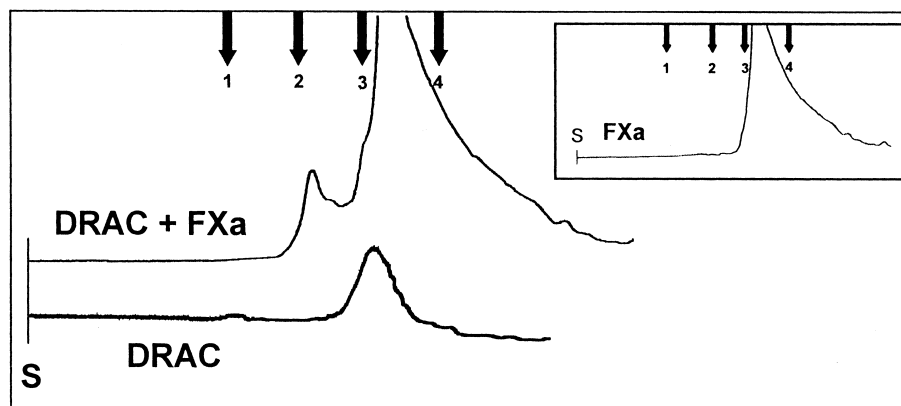


Fig. 1. Molecular exclusion HPLC profile of draculin (DRAC) and a mixture of Draculin and FXa (DRAC+FXa). The arrows indicate elution time of markers: 1, catalase 232 kDa; 2, lactate dehydrogenase 140 kDa; 3, phosphorylase *b* 94 kDa; 4, albumin 67 kDa. The inset corresponds to the HPLC profile of FXa under the same experimental conditions.

3. Results

3.1. HPLC of the Draculin–FXa complex

As shown in Fig. 1, incubation of Draculin with an excess of FXa results in a molecular exclusion pattern in which a new protein peak, with molecular mass in the range of 130–150 kDa is clearly apparent (arrow), suggesting a 1:1 complex between Draculin and FXa, which is not easily dissociated under the experimental conditions used.

3.2. Effect of factor Xa on Draculin

Aliquots from a mixture of Draculin (400 nM) and FXa (200 nM) were taken after 24, 48 and 90 h of incubation. Western blotting analysis shows no change in the apparent molecular mass of Draculin. Furthermore, degradation products reacting with anti-Draculin antibodies were not observed (not shown). These results indicate that Draculin is not a substrate for FXa.

3.3. Effect of Draculin on the progress curve for the amidolytic activity of FXa

Preincubation of FXa with Draculin inhibits the catalytic activity of the enzyme in a dose-dependent manner at concentrations that are comparable to the

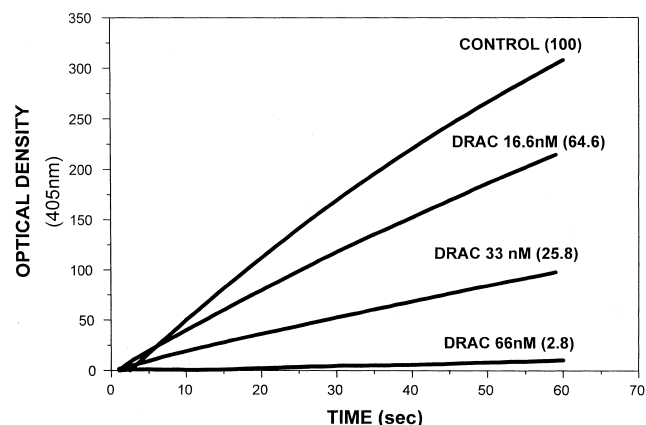


Fig. 2. Progress curves showing the concentration-dependent effect of Draculin on the amidolytic activity of FXa (10 nM). Values in parentheses represent residual activity of FXa (percentage). S-2337 (400 μ M) was used as substrate. Other conditions as described in Section 2.

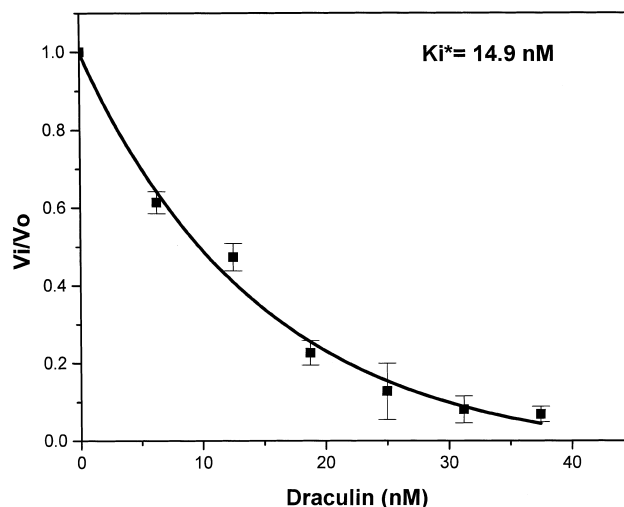


Fig. 3. Determination of the intrinsic $K_i(K_d)$ for Draculin. The inhibitory activity is expressed as the ratio of the inhibited rate to uninhibited rate (fractional activity). FXa concentration was 10 nM. Substrate used: S-2337 (400 μ M). Experimental data (three determinations) adjusted to Morrison's equation (line) yield a K_i value of 14.8 ± 1.5 nM.

total enzyme concentration (Fig. 2). Increasing the incubation time does not change the degree of inhibition (results not shown). For practical reasons 1 min incubation time was routinely used. Initial rates were obtained from the linear (< 25 s) part of the progress curve in order to avoid the effect of substrate depletion on the linearity of the progress curve also observed in the control assay. The apparent lag phase shown in the figure at the highest Draculin concentration was not observed in repeated experiments ($n = 10$).

From the progress curves shown in Fig. 2, inhibited (V_i) and uninhibited (V_o) initial rates were calculated and then fitted by nonlinear regression to the tight-binding equation [20]:

$$\frac{V_i}{V_o} = \frac{1}{2E_t} \left[(E_t - I_t - K_i^*) + \sqrt{(I_t + K_i^* - E_t)^2 + 4K_i^*E_t} \right]$$

where V_i/V_o represents the fractional activity of FXa (inhibited velocity divided by uninhibited velocity), E_t is the total concentration of enzyme, I_t is inhibitor concentration and K_i^* is the dissociation constant (Fig. 3). The value of $K_i(K_d)$ for the interaction of Draculin and FXa calculated from this equation is 14.8 ± 1.5 nM.

3.4. Measurement of rate constants for formation and dissociation of Draculin–FXa complex

Fig. 4 shows the effect of preincubation of FXa with Draculin (Xa+Drac+S curve) and the effect of Draculin addition after 20 s of the start of the reaction (Xa+S+Drac curve). Draculin added to FXa in the presence of excess substrate ($3K_m$) very rapidly induces inhibition of the catalytic activity of the enzyme (Fig. 4, Xa+S+Drac curve). The level of the steady state of residual activity is dependent on Draculin concentration as shown in Fig. 2, but is not dependent on the order of addition of Draculin. Prior incubation of factor Xa with Draculin, for longer periods of time, as shown in Fig. 4, does not increase the final degree of inhibition attained. The inhibition progress curves, from experiments presented in Fig. 4 (Xa+S+Drac curve), were fit to a single exponential function to obtain the half-time ($t_{1/2}$) for the inactivation of FXa at different concentrations of Draculin. The values for k_{obs} were obtained from $k_{obs} = 0.693/t_{1/2}$ [19]. A plot of k_{obs} vs. Draculin concentration, fitted to a hyperbolic equation (Fig. 5),

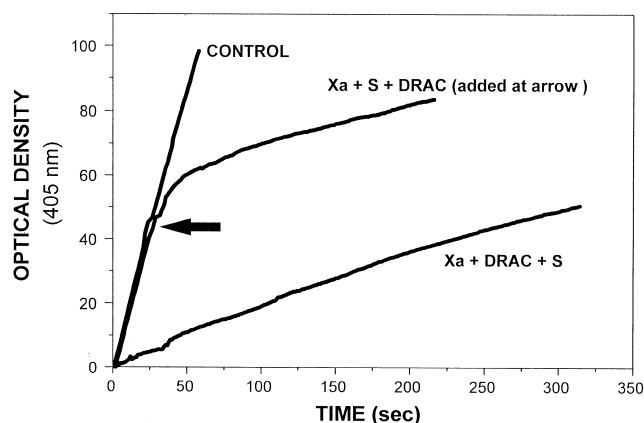


Fig. 4. Time-dependence of the effect of Draculin on the amidolytic activity of FXa. FXa = 1 nM; Draculin = 10.4 nM; substrate used was $\text{CH}_3\text{OCO-D-CHG-Gly-Arg-pNA} = 400 \mu\text{M}$. Steady-state residual activity is independent of the order of addition and time of incubation of Draculin. Experimental conditions as described in Section 2.

shows a linear, concentration-dependent portion followed, at Draculin concentrations higher than 50 nM, by a concentration-independent phase; from the linear part of the plot (concentration-dependent), the

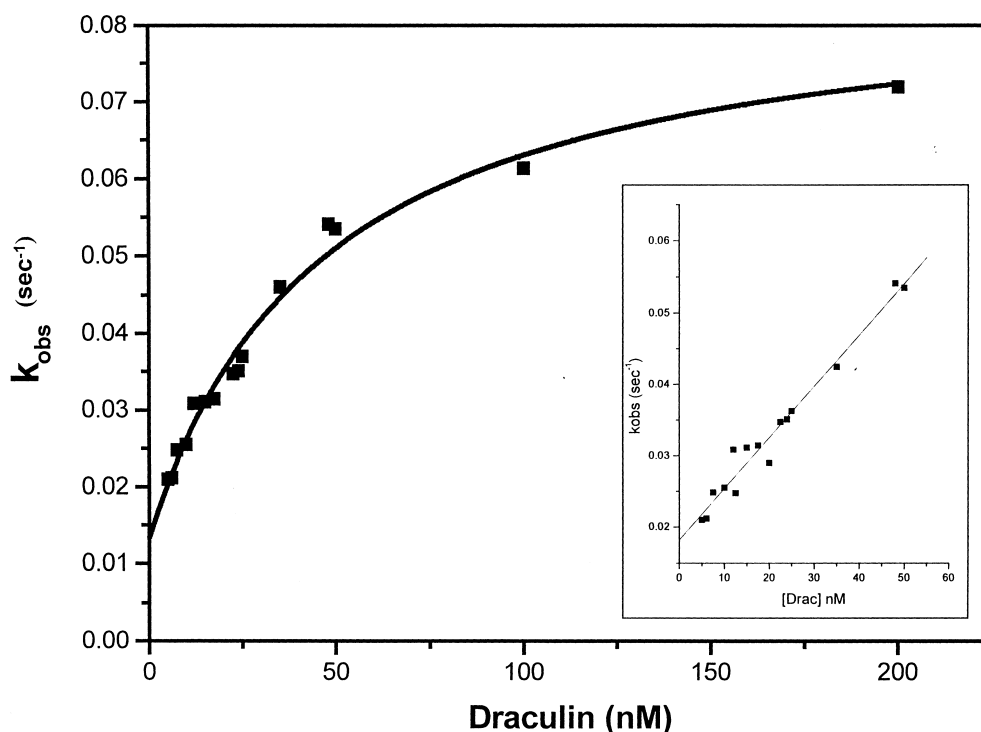


Fig. 5. Dependence of k_{obs} on Draculin concentration. Values of k_{obs} were obtained from experiments as shown in Fig. 4, as described in Section 3, at various Draculin concentrations. The association rate constant k_{on} was calculated from the slope ($k_{on} = k_{obs}/[\text{Draculin}] = 1.117 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$). Value for k_{off} , obtained from the y-intercept = k_{off} is $15.388 \times 10^{-3} \text{ s}^{-1}$.

relation $k_{\text{obs}} = k_{\text{on}} [\text{Draculin}] + k_{\text{off}}$ allows the calculation of $k_{\text{on}} = 1.117 (\pm 0.169) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and $k_{\text{off}} = 15.388 (\pm 1.672) \times 10^{-3} \text{ s}^{-1}$. K_{d} , estimated from $k_{\text{off}}/k_{\text{on}}$, equals $13.76 \times 10^{-9} \text{ M}$, which is in accordance with the K_{i} calculated from fitting the fractional activity ($V_{\text{i}}/V_{\text{o}}$) data to the tight-binding equation.

Double reciprocal plot of initial rates of conversion of the low- K_{m} substrate $\text{CH}_3\text{OCO-D-CHG-Gly-Arg-pNA}$ at different Draculin concentrations yields the typical pattern of noncompetitive inhibition (Fig. 6).

3.5. Effect of Draculin on the binding of the active-site directed probe p-aminobenzamidine to FXa

The fluorescent molecule pAB binds to the active site of serine proteases displaying, upon binding, an increase in fluorescence yield [13]. pAB can be displaced, with concomitant decrease of fluorescence, by other compounds that bind to the enzyme active site (i.e., benzamidine, AT-III for FXa) [14]. As shown in Fig. 7A, addition of pAB to FXa alone yields a fast increase in fluorescence and subsequent addition of BA produces a rapid decrease in fluorescence to almost the baseline level. When pAB is added to the mixture of FXa and Draculin (Fig. 7B), a similar

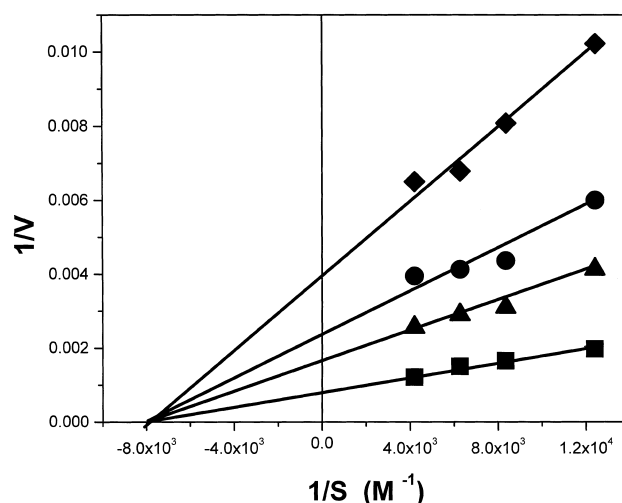


Fig. 6. Double reciprocal plot indicative of the noncompetitive behavior of Draculin. FXa (4 nM); substrate used was $\text{CH}_3\text{OCO-D-CHG-Gly-Arg-pNA}$ ($K_{\text{m}} = 104 \mu\text{M}$). (■) Control; (●) Draculin 21.36 nM; (▼) Draculin 32.18 nM; (◆) Draculin 41.20 nM. Solid lines represent the best fit of the data.

increase in fluorescence as in the control (Fig. 7A) is obtained. Furthermore, addition of Draculin to the pAB–FXa complex, at a concentration that completely inhibits FXa activity (Fig. 7C), produces at most a 14% decrease in the fluorescence yield of the pAB–FXa complex. Increasing time of incubation of

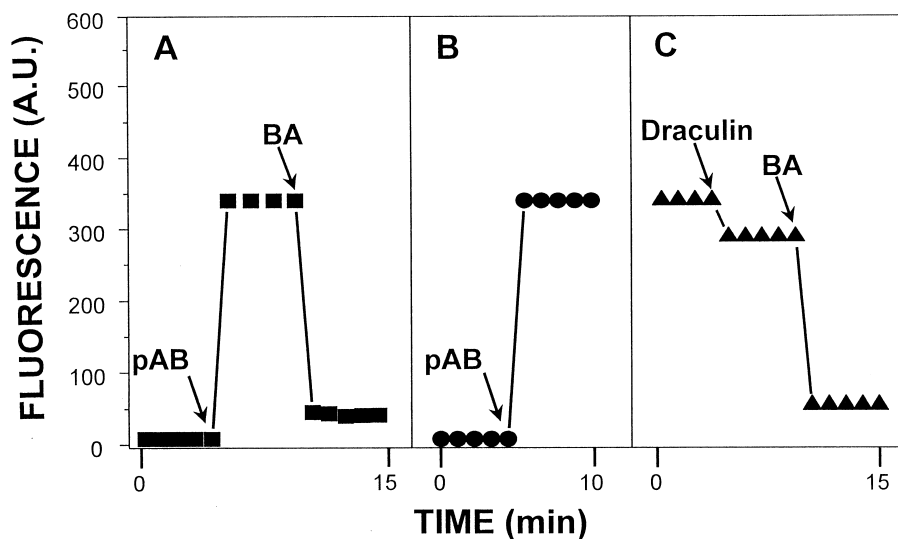


Fig. 7. Effect of Draculin on the binding of the active-site directed probe pAB. (A) pAB (75 μM) added to FXa (1 μM) alone; addition of benzamidine (1.13 mM) displaces pAB and decreases fluorescence to basal level. (B) Addition of pAB to FXa preincubated with Draculin (7 μM). (C) Addition of Draculin to the pAB–FXa complex decreases by < 14% the fluorescence yield; addition of benzamidine to the mixture returns the fluorescence to the initial level.

Table 1
Molecular mass and kinetic parameters of Draculin and other natural anti-FXa polypeptides

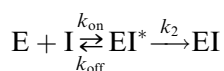
FXa Inhibitor	Molecular mass (kDa)	K_i ($\times 10^{-9}$ M)(K_d)	$k_{on} \times 10^6$ ($M^{-1} s^{-1}$)	k_{off} (s^{-1})	Ref.
Draculin	88.5	13.76–14.80	1.117	15.833×10^{-3}	
Antistasin	15	0.31–0.62	1.2	$5.1\text{--}5.7 \times 10^{-4}$	[2]
rTAP	6.8	0.18	2.85	0.554×10^{-3}	[22]
Ecotin	16	0.054	1.35	6.5×10^{-5}	[6,7]
AcAP	8.7	0.323	–	–	[8]

FXa with Draculin does not produce any further changes. The same figure shows that addition of BA to this mixture decreases the fluorescence yield as in the absence of Draculin. These results show that Draculin neither impairs binding of pAB to the enzyme, nor affects its displacement by BA. This implies, in agreement with the noncompetitive kinetic pattern, that Draculin–FXa and Draculin–FXa–[S] complexes can be simultaneously formed. The slight decrease in fluorescence yield observed for the binding of pAB to the Draculin–FXa complex might be due to subtle conformational changes in the complex that affect the quantum yield of fluorescence.

4. Discussion

Draculin, the anticoagulant glycoprotein isolated from vampire bat (*Desmodus rotundus*) saliva, is a single-chain polypeptide with molecular mass of 88.5 kDa that selectively inhibits FIXa and FXa [9]. The Lineweaver–Burk pattern, as well as the lack of interference of Draculin with the active-site ligand *p*-aminobenzamidine, clearly indicate that Draculin acts as a noncompetitive inhibitor of FXa. The facts that Draculin establishes a relatively rapid equilibrium with FXa, in a range of similar concentrations of enzyme and inhibitor, and that no lag phase is observed at any of the Draculin concentrations assayed, allows Draculin to be considered within the category of tight-binding inhibitors [21]. The lack of cleavage of Draculin by FXa, even after prolonged periods of time (up to 90 h) agrees with the noncompetitive mode of inhibition, a fact that could be potentially advantageous for the vampire bat feeding. We have previously shown that Draculin bound to FXa immobilized on AffiGel is not eluted

even after extensive washings of the matrix [9], suggesting that the FXa–Draculin complex can be reasonably considered as irreversible, under the experimental conditions used. These results, in addition to those suggesting that Draculin forms an irreversible complex with FXa, can be explained in part through the formation of an inactive Draculin–FXa complex by a two-step mechanism of the type



where formation of EI^* is concentration-dependent, while transformation of EI^* into EI is concentration-independent and essentially irreversible. This result is in agreement with our present observation that the Draculin–FXa complex is stable enough to withstand separation through a molecular exclusion column, and supports the assumption of irreversibility for the second step proposed in our kinetic scheme. Since the formation of EI is an essentially irreversible step, the value for $k_{obs} = 0.072 s^{-1}$, obtained at the highest Draculin concentration, closely represents k_2 , the rate constant for the concentration independent step.

These facts distinguish Draculin from other known natural anti-Xa polypeptides [2–4,7,8], which behave as competitive, slow, tight-binding inhibitors and, in the case of Ecotin and Antistasin, are reported to be slowly cleaved by the enzyme they inhibit. In Table 1, we compare the molecular mass and kinetic parameters obtained for the interaction of FXa and Draculin with those published for Antistasin, Ecotin, rTAP and AcAP. Although k_{on} is in the same range of values reported for the other inhibitors, the fact that k_{off} is one to two orders of magnitude faster makes Draculin a weaker inhibitor (K_d in the nM range). The noncompetitive behavior of Draculin may be a plausible explanation for its dual, nonin-

terfering inhibitory action on both FIXa and FXa [9], and perhaps a biological advantage to the blood-feeding need of the vampire bat. Structure–activity relationship studies, under way in our laboratory, should help to identify portion(s) of the molecule relevant to its interaction with activated factors IX and X, which may lead to the design of new anti-coagulant compounds with distinctive inhibitory mechanisms.

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