Isolation, Cloning and Structural Characterisation of Boophilin, a Multifunctional Kunitz-Type Proteinase Inhibitor from the Cattle Tick

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Abstract

Inhibitors of coagulation factors from blood-feeding animals display a wide variety of structural motifs and inhibition mechanisms. We have isolated a novel inhibitor from the cattle tick Boophilus microplus, one of the most widespread parasites of farm animals. The inhibitor, which we have termed boophilin, has been cloned and overexpressed in Escherichia coli. Mature boophilin is composed of two canonical Kunitz-type domains, and inhibits not only the major procoagulant enzyme, thrombin, but in addition, and by contrast to all other previously characterised natural thrombin inhibitors, significantly interferes with the proteolytic activity of other serine proteinases such as trypsin and plasmin. The crystal structure of the bovine α-thrombin-boophilin complex, refined at 2.35 Å resolution reveals a non-canonical binding mode to the proteinase. The N-terminal region of the mature inhibitor, Q16-R17-N18, binds in a parallel manner across the active site of the proteinase, with the guanidinium group of R17 anchored in the S1 pocket, while the C-terminal Kunitz domain is negatively charged and docks into the basic exosite I of thrombin. This binding mode resembles the previously characterised thrombin inhibitor, orthonhodrin which, unlike boophilin, is composed of two distorted Kunitz modules. Unexpectedly, both boophilin domains adopt markedly different orientations when compared to those of orthonhodrin, in its complex with thrombin. The N-terminal boophilin domain rotates 9° and is displaced by 6 Å, while the C-terminal domain rotates almost 6° accompanied by a 3 Å displacement. The reactive-site loop of the N-terminal Kunitz domain of boophilin with its P1 residue, K31, is fully solvent exposed and could thus bind a second trypsin-like proteinase without sterical restraints. This finding explains the formation of a ternary thrombin-boophilin-trypsin complex, and suggests a mechanism for prothrombinase inhibition in vivo.

Introduction

Kunitz-type domains are common structural and functional elements found in extracellular proteins [1], which are usually associated with inhibition of trypsin-like serine proteinases [2]. Kunitz domains appear not only in single-domain proteins such as the paradigmatic member of the family, bovine pancreatic trypsin inhibitor (BPTI) [3], but also in multiple tandem repeats (e.g., the three consecutive domains that build up the major inhibitor of the coagulation cascade, tissue factor pathway inhibitor/TFPI) [4].

The crystal structure of the trypsin-BPTI complex [5] and ensuing structural analysis of other proteinase-Kunitz inhibitor pairs [6–10] provided major insights into the mechanism of serine proteinase inhibition, as well as into our current understanding of peptide bond cleavage by serine proteinases. In these enzyme-inhibitor complexes, the Kunitz domain inserts a protruding, disulfide-stabilised loop-the reactive-site loop-into the active-site cleft of its cognate proteinase. Essential elements of this binding mode, nowadays termed canonical, are that (i) the reactive-site loop adopts a substrate-like conformation by forming a short β-strand that aligns anti-parallel to proteinase residues S214–G216, with (ii) its P1 K/R residue occupying the S1 specificity pocket of the enzyme [11] (The chymotrypsinogen)-based numbering system is used throughout this work, as well as the Schechter and Berger nomenclature, in which substrate/inhibitor residues are denoted Pα, ..., P1, P′1, ..., Pα′, from N- to C-terminal, where PαPα′ is the scissile peptide bond. The corresponding proteinase subsites that accommodate these residues are accordingly termed Sα, ..., S1, S′1, ..., Sα′).

Hematophagous animals interfere with several mechanisms that support homeostasis in their preys. In particular, these parasites strictly require inhibition of blood clot formation to allow successful feeding and digestion. Thrombin (EC 3.4.21.5), as the ultimate proteinase of the coagulation cascade [12], represents
an attractive and perhaps obligatory target for these animals. The proteinase possesses two characteristic surface areas enriched in basic residues located far from the catalytic centre, termed anion-binding exosites. Exosites I and II allow high affinity interactions with various macromolecular substrates and receptors. For instance, exosite I mediates selective binding of fibrinogen and protease activated receptors (PARs), as well as the endothelial receptor, thrombomodulin, while exosite II recognises glycosaminoglycans such as heparin and the platelet receptor GP Ib-IX-V (for a recent review on the role of thrombin exosites, see ref. [13]).

Previously solved crystal structures of thrombin in complex with unrelated inhibitors derived from insects (e.g., rhodniin [14] and triabin [15]) or leeches [16,17] have shown that exosites are also critical for inhibitor binding.

In spite of the large number of serine proteinases targeted by Kunitz domains, they were long considered ineffective as thrombin inhibitors. In fact, BPTI inhibits thrombin with a \( K_i \) value of \( \sim 100 \) \( \mu \)M, ten orders of magnitude worse than trypsin [18,19]. The crystal structure of human \( \alpha \)-thrombin revealed a major reason for this observation: the reactive-site loop of a canonically binding Kunitz inhibitor would severely clash with the narrow active-site cleft of the proteinase, in particular with the unique insertion Y60A-W60D [20]. Another factor that disfavours binding of Kunitz inhibitors to thrombin is the presence of a glutamate at position 192, a position commonly occupied by a Gln residue. Accordingly, both the deletion of the triplet P60B-W60D and the single E192-Q mutation notably enhance thrombin affinity for BPTI and TFP1 [21,22].

The crystal structure of the (E192Q)thrombin-BPTI complex revealed large displacements of the insertion 60-loop, of up to 8 \( \AA \) for the Cz atom of W60D [23]. This finding raised the following speculation about the role of thrombomodulin: binding to exosite I of the proteinase could induce repositioning of active site residues and surrounding loops, to allow processing of a substrate that is not cleaved by free thrombin, the anticoagulant protein C. Although the crystal structure of the thrombin-thrombomodulin complex did not reveal noticeable differences in the active site region of bound thrombin, when compared to other reported structures [24], it still remains theoretically possible that stronger exosite-binders could allosterically elicit large distortions at the catalytic centre.

The only thrombin inhibitor from the Kunitz family structurally characterised to date, ornithodorin from the soft tick Ornithodorus moubata, is comprised of two highly distorted Kunitz modules, none of which would be able to bind a serine proteinase according to the canonical mechanism [25]. Instead, ornithodorin docks with its C-terminal residues to bind across the active-site cleft. This N-terminal peptide adopts a parallel conformation with regard to \( \beta \)-strand S214-G216; the resulting non-substrate-like binding mode is thus similar to that of leech-derived inhibitors hirudin [16] and haemadin [17]. A non-canonical binding mode was also described for tick anticoagulant peptide (TAP), an *O. moubata* factor Xa (FXa) inhibitor [26].

The cattle tick *Boophilus microplus* (Acari: Ixodidae; recently reclassified as *Rhipicephalus microplus*) is one of the most widely distributed parasites of farm animals, causing an important economic impact both due to blood and milk losses and leather damage, and because it is a vector of major protozoan pathogens [27]. Proteinase inhibitors have long been recognised in eggs and larvae of *B. microplus*, and target trypsin, chymotrypsin, elastase, and plasma kallikrein [28–33]. Interestingly, some inhibitors were shown to prolong the activated partial thromboplastin time (APTT) for bovine blood. At most partial sequence data has been reported for these inhibitors, and their potential effect on blood coagulation remains to be clarified. More recently, the antimicrobial single-domain inhibitor, ixodulin, was isolated from the hemocytes of *B. microplus* and shown to target both elastase and chymotrypsin [34], while genomic analysis revealed the presence of additional Kunitz-type inhibitors that are similar e.g. to TFP1 [35,36].

Here we report the isolation, preliminary functional characterisation, cDNA cloning, and heterologous expression of a novel thrombin inhibitor from *B. microplus*, which we have termed boophilin. In contrast to all previously discovered natural thrombin inhibitors, boophilin potently inhibits additional trypsin-like serine proteinases, including trypsin and plasmin. Besides, and in line with these functional observations, its amino acid sequence is more closely related to canonical Kunitz inhibitors such as BPTI than to ornithodorin. These findings, in turn, suggested that boophilin could employ the energy liberated upon occupancy of exosite I to “loosen” thrombin’s 60-loop, thus allowing insertion of its reactive-site loop in a canonical manner, and provoked a structural investigation of its mechanism of thrombin inhibition. However, the crystal structure of the bovine thrombin-boophilin complex, refined at 2.35 \( \AA \) resolution, reveals a non-canonical, ornithodorin-like binding mode; the reactive-site loop of the N-terminal domain remains thus freely accessible to a second trypsin-like proteinase. Surprisingly, all elements involved in thrombin-inhibitor interactions occupy different relative positions when compared to the corresponding regions in the thrombin-ornithodorin complex.

**Results**

**Isolation of a thrombin inhibitor present in engorged *B. microplus* ticks**

Approximately 150 g of engorged ticks were used for inhibitor purification. A single peak containing thrombin inhibitory activity was detected on ion-exchange chromatography, and further purified by affinity chromatography on a thrombin-Sepharose column. This two-step purification protocol yielded about 50 \( \mu \)g of an essentially pure protein, as judged by PAGE analysis, which we have termed boophilin. This inhibitor displays an apparent molecular mass (M) of \( \sim 23 \) kDa (Supplementary Figure S1), as estimated by SDS-PAGE under reducing conditions, which almost doubles the value determined by mass spectrometry (13.9 kDa; Supplementary Figure S1). The anomalous migration in SDS-polyacrylamide gels appears to be related to the acidic character of the protein (calculated pI 4.1; see below), which could interfere with SDS binding. We also note that the unreduced protein migrates faster than reduced boophilin, suggesting the presence of a disulfide-knotted domain(s).

Boophilin was shown to double the thrombin clotting time of human plasma at an effective concentration of about 0.1 \( \mu \)M (mean of four determinations). Boophilin also showed a significant effect on the prothrombin time (effective concentration 0.4 \( \mu \)M; mean of four determinations), as well as a weaker effect on the activated partial thromboplastin time (effective concentration 1.7 \( \mu \)M, mean of two determinations). Assuming that the purified inhibitor binds bovine thrombin with tight-binding kinetics, we determined an apparent \( K_i \) of 1.8 nM. Further, we could show formation of a stable 1:1 thrombin-boophilin complex in solution (Figure 1A). A free active site was required for complex formation, as no stable proteinase-inhibitor complex could be detected upon incubation with PPACK-inhibited thrombin (data not shown). These findings were confirmed by surface plasmon resonance experiments (Biacore); boophilin showed high affinity for free human \( \alpha \)-thrombin, but bound only weakly to PPACK-thrombin or prethrombin-2 (not shown).
Boophilin inhibits other serine proteinases in addition to thrombin

In striking contrast to other characterised thrombin inhibitors, boophilin could block the amidolytic activity of other trypsin-like serine proteinases, most notably trypsin and plasmin (Table 1). We examined trypsin inhibition in more detail and determined that boophilin inhibits this serine proteinase according to a slow-binding mechanism, with a second order rate constant \(k_2/K_i\) of \((3.08 \pm 0.60) \times 10^5\) M\(^{-1}\)s\(^{-1}\) (mean of four determinations). In addition, we corroborated formation of a stable 1:1 trypsin-boophilin complex in solution (Figure 1B). By contrast, the activity of other trypsin-like serine proteinases, and in particular of FXa, was only marginally affected (Table 1).

Boophilin is comprised of two Kunitz-type domains

Edman degradation of affinity-purified boophilin failed to reveal a signal for any amino acid residue, suggesting that the N-terminus of the inhibitor was blocked. However, sequences of internal boophilin regions could be obtained form chemically or enzymatically generated peptides. Comparison of these sequences against the Swiss-Prot database revealed strong similarities to Kunitz-type proteinase inhibitors from different species, in particular to the prototypic member of the family, BPTI (Supplementary Table S1; see also below). In light of the molecular mass determined with MALDI-MS (matrix-assisted laser desorption/ionisation mass spectrometry), this partial sequence data strongly suggested that boophilin comprised two Kunitz-type domains (M\(_r\) < 6,500 Da) connected by a short linker.

To continue structural and functional characterisation of boophilin, we decided to clone and express the recombinant inhibitor. For this purpose, we constructed a cDNA library of unfed B. microplus containing 1.8 \(\times\) 10\(^6\) independent clones, with inserts ranging from 0.5 to 4.0 kb in size. Degenerate oligonucleotides Lys-C11Fwd and Lys-C15Rev were designed (Supplementary Table S2), exploiting the presence of a triplet of aromatic residues between the second and third cysteine residue in each of the two boophilin Kunitz-type domains. These primers were used to amplify a single, 300-bp cDNA fragment by PCR on randomly primed cDNA derived from poly(A\(^+\)) RNA of engorged ticks. The size of this PCR product is in good agreement with the figure expected for a DNA fragment coding for the sequenced boophilin fragment (see above). A band of the same size was observed when performing PCR reactions on the cDNA library phage stock, confirming boophilin expression in unfed animals as well.

Screening of the cDNA library with the labelled amplified fragment allowed identification of six independent positive clones, which were isolated and sequenced. These independent cloning events confirmed the presence of two boophilin variants (in the following termed G2 and H2), as suggested by the protein sequencing results. The complete nucleotide and deduced amino acid sequence of variant G2 is presented in Figure 2. The DNA sequences of the two cloned boophilin variants differ at twelve independent positions. All of these differences arise from point mutations and result in six amino acid exchanges in the encoded proteins: two at positions 3 and 4 of the propeptide, and four in the mature protein. However, these substitutions are conservative (D66→R in the N-terminal Kunitz domain, S78→N and G82→S in the linker region, and R110→Q in the C-terminal domain), and are thus not expected to interfere with the inhibitory potential of boophilin. Rather, they could reflect the adaptation to different
Table 1. Inhibition profile of boophilin

| Enzyme   | nBoophilin | rBoophilin | rBoophilin (C-term) |
|----------|------------|------------|---------------------|
| Trypsin  | 97         | 98         | 80                  |
| Factor Xa| 10         | 0          | 0                   |
| Factor XIIa| n.d.      | n.d.      | 0                   |
| Plasma kallikrein | 66   | 80         | 0                   |
| Factor VIlla| n.d.     | n.d.      | 0                   |
| Plasmin  | 87         | 95         | 25                  |
| u-PA     | 16         | 0          | 17                  |
| sc-tPA   | n.d.       | 4          | 2                   |
| Tryptase | n.d.       | n.d.       | 8                   |

Inhibitor samples were incubated with the nine serine proteinases listed, and the residual activity was measured after adding proteinase-specific chromogenic substrates, as follows: Pefachrome tPA (final substrate concentration 0.18 and 0.54 mM, respectively) to trypsin (final enzyme concentration 90 ng/ml) or sc-tPA (1.9 μg/ml); Chromozym X (0.36 mM) to FXa (0.11 U/ml); Pefachrome FXIIa (0.18 mM) to FXIIa (1.8 μg/ml); Chromozym PK (0.36 mM) to plasma kallikrein (0.52 μg/ml); Chromozym tPA (0.73 mM) to FVIIIa (2.2 μg/ml); Chromozym PL (0.18 mM) to plasmin (91 μg/ml); Pefachrome uPA (0.16 μl) to u-PA (154 U/ml) and Chromozym TH (0.18 mM) to tryptase (0.11 μg/ml). Reactions were allowed to proceed for 2-6 min, and the effect of boophilin was estimated by setting the activity obtained without inhibitor as 100%. n.d., not determined. The concentrations used for boophilin samples were: native (n) inhibitor, 3.53 μM recombinant (r) inhibitor (full-length), 2.36 μM recombinant inhibitor (C-terminal domain), 15.2 μM.

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preys or the response to immunological pressure, as observed in other blood-feeding animals.

The sequences of mature boophilin and of another related, recently reported thrombin inhibitor, amblin [37], are compared to the previously characterised ornithodorin and to BPTI in Figure 3. It is immediately apparent that both boophilin domains are much more closely related to BPTI and other canonical Kunitz modules than to ornithodorin. Twenty-six residues of the N-terminal and 22 residues of its C-terminal domain are identical to BPTI, whereas the corresponding domains in boophilin and ornithodorin share only 12 or 13 identical residues. Furthermore, cysteine residue spacing is exactly conserved in boophilin and BPTI. Most relevant for serine proteinase inhibition, the sequence of the reactive-site loop in the N-terminal domain (G229-I-C-K ↓ A-I-I-P35; K31 corresponds to the P1 residue) closely matches the corresponding G12-P-C-K ↓ A-R-I-I9 sequence in BPTI and other Kunitz inhibitors, and the secondary binding loop (F91-C54) is even better conserved. These sequence similarities between boophilin and BPTI contrast with the multiple insertions and deletions found in ornithodorin (Figure 3), which altogether hinder its binding to serine proteinases according to the canonical mechanism [25].

Recombinant expression of boophilin and further functional characterisation

A cDNA fragment coding for the full-length mature protein (residues Q16 to M142, variant H2) was amplified by PCR and subcloned into the pRBI-DsbC expression vector, as a fusion construct with the OmpA periplasmic signal sequence (oligonucleotides used for subcloning are given in Supplementary Table S2). Escherichia coli DH5α cells were transformed with the expression construct and used for heterologous expression of recombinant boophilin, which was found to be correctly processed and active in the periplasmic space of the bacteria (not shown). The recombinant product was purified to homogeneity by anion-exchange chromatography on Q-Sepharose FF and ResourceQ columns, and authenticity was verified by N-terminal sequencing.

To confirm production of a correctly folded inhibitor we have verified the inhibitory effect of recombinant boophilin on blood coagulation concentrations needed for doubling clotting times in the absence of the inhibitor: thrombin time, 0.045 μM; prothrombin time, 0.160 μM; APTT, 0.880 μM, as well as its ability to inhibit thrombin (Ki = 3.4 nM). Next, we verified the inhibition of the two other trypsin-like serine proteinases (trypsin and plasmin), as well as a partial effect on plasma kallikrein (80% inhibition at 2.36 μM, still 32% inhibition at 0.47 μM; compare Table 1). Finally, we have detected a weak inhibitory effect on FVIIa (57% inhibition at 2.36 μM). In contrast, other serine proteinases (FXa, FXIIa, uPA, sc-tPA, and tryptase) were not inhibited by boophilin. The second-order inhibition constants against trypsin and plasmin, determined using recombinant boophilin, are (3.84±0.73)×105 M⁻¹s⁻¹ (mean of three determinations) and (2.59±0.22)×10⁵ M⁻¹s⁻¹ (mean of four determinations), respectively, and thus similar to the values reported for BPTI [18,38]. All inhibitory properties of recombinant boophilin are similar to those observed for the inhibitor isolated from natural sources. Small differences observed are likely to be accounted for by the presence of a mixture of boophilin isoforms, in the native material.

By contrast, the isolated C-terminal boophilin domain, serendipitously obtained by cleavage of the A83-D84 peptide bond in the interdomain linker of recombinant boophilin by an endogenous protease of E. coli, showed essentially no effect on either thrombin time, prothrombin time or partial thromboplastin time at a concentration of 1 μM. Only at a rather high protein concentration of 20 μM, a marginal increase (30%) in thrombin time was observed. In addition, none of the serine proteinases tested was significantly inhibited by the isolated boophilin domain (Table 1). Clearly, the sequences of mature boophilin and of another related, recently reported thrombin inhibitor, amblin [37], are compared to the previously characterised ornithodorin and to BPTI in Figure 3. It is immediately apparent that both boophilin domains are much more closely related to BPTI and other canonical Kunitz modules than to ornithodorin. Twenty-six residues of the N-terminal and 22 residues of its C-terminal domain are identical to BPTI, whereas the corresponding domains in boophilin and ornithodorin share only 12 or 13 identical residues. Furthermore, cysteine residue spacing is exactly conserved in boophilin and BPTI. Most relevant for serine proteinase inhibition, the sequence of the reactive-site loop in the N-terminal domain (G229-I-C-K ↓ A-I-I-P35; K31 corresponds to the P1 residue) closely matches the corresponding G12-P-C-K ↓ A-R-I-I9 sequence in BPTI and other Kunitz inhibitors, and the secondary binding loop (F91-C54) is even better conserved. These sequence similarities between boophilin and BPTI contrast with the multiple insertions and deletions found in ornithodorin (Figure 3), which altogether hinder its binding to serine proteinases according to the canonical mechanism [25].

Recombinant boophilin retains the capability to interact with other serine proteinases

The functional evidence presented above, combined with structural information for thrombin-ornithodorin [25] and thrombin-n/E192Q-BPTI complexes [23], immediately suggested two possible modes for boophilin binding to cognate thrombin (Figure 4A). In both cases, the highly acidic boophilin C-terminal domain (11 aspartate/glutamate residues outweigh four basic residues) would interact with thrombin’s positively charged exosite I. However, the two models differ with regard to the region of the N-terminal domain that blocks the active site of the protease. Binding in a canonical manner, i.e. via reactive-site loop requires major rearrangements of loops surrounding the active centre, and in particular of the 60-loop. By contrast, in the ornithodorin-like conformations only the N-terminal peptide of the inhibitor interacts with thrombin active site, which is essentially unmodified compared to the free enzyme. Preliminary modelling experiments indicated that both conformations could be plausibly adopted in the binary thrombin-boophilin complex, given the considerable length of the interdomain linker.

To discriminate between these opposite binding modes, we reasoned that the reactive-site loop of the bound inhibitor would only be exposed in the ornithodorin-like conformation to allow
Figure 2. Complete nucleotide sequence of the cDNA encoding for boophilin variant G2 and predicted amino acid sequence. The coding region is shaded in light green, the start codon in dark green, the stop codon in red, the polyadenylation signal in orange, and the short poly(A) tail in yellow. The deduced amino acid sequence of pro-boophilin is shown in red below the nucleotide sequence, in one-letter code. An orange line indicates the fragment amplified by PCR. The in-frame stop codon that precedes the start codon for pro-boophilin is shaded blue. The deduced amino acid sequence of boophilin (142 residues) would correspond to a polypeptide with a higher molecular mass than that experimentally determined by MALDI-MS. However, if one considers the presence of a 15-residue pro-peptide, the resulting mature inhibitor would have a theoretical mass of 13,950 Da, in reasonable agreement with the experimentally determined value of 13,964 Da. (Notice that no putative N-glycosylation sites are present in the boophilin sequence). Further, the mature protein starts with a glutamine residue that could spontaneously cyclise to pyrrolidone carboxylic acid (5-oxo-proline). This modification would explain the blocked N-terminus observed in native boophilin, and has previously been reported in other Kunitz inhibitors such as an BPTI variant from bovine lung [74]. Open triangles indicate the positions where discrete differences exist in the amino acid sequences of the two boophilin variants.

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Figure 3. The two boophilin domains are more closely related to canonical Kunitz modules than to ornithodorin. Comparison of the amino acid sequence of mature boophilin with three other two-domain inhibitors, ornithodorin, amblin, and bikunin; the unique C-terminal extension in amblin has been omitted for clarity. Also included in the alignment are the sequences of BPTI and of the closest related human Kunitz modules structurally characterised so far (two copies, one aligned with each Kunitz module of boophilin). The second and third TFPI domains (1TFX [6] and 1RH [75], respectively), the single Kunitz domain of APP (1AAP [39], and the C-terminal Kunitz domain of type VI collagen (2KNT) [76]. Numbers refer to the full-length sequence of boophilin. Residues conserved throughout are white with red shading; residues identical to boophilin are white with blue shading; conservatively replaced residues are shaded green. The four major antigenic determinants in boophilin are boxed. The P1 residues within the reactive-site loops are indicated with black arrowheads; open arrowheads point to boophilin/ornithodorin residues involved in important contacts with thrombin exosite I. Notice that several exosite-interacting residues are conserved or conservatively replaced in boophilin, while they are mostly non-conservatively replaced in amblin (e.g., E130→K, L137→K).

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targeting of a second serine proteinase. This possibility was analysed using trypsin as a probe. Indeed, we could demonstrate formation of a ternary thrombin-boophilin-trypsin complex in solution (Figure 4B), in agreement with a non-canonical binding mode. We conclude that the N-terminal boophilin domain can simultaneously engage thrombin through its N-terminal peptide, and a second trypsin-like serine proteinase in a canonical manner.

Thrombin structure is not altered in its complex with boophilin

To verify the non-canonical mechanism of thrombin inhibition by boophilin, we decided to solve the three-dimensional structure of the equimolar bovine thrombin-boophilin complex. The complex crystallises as a dimer in the asymmetric unit (depicted in Figure 5). This non-crystallographic dimer is maintained by a small number of water-mediated polar interactions, mostly connecting opposite thrombin molecules. The small size of buried surface (∼400 Å²), as well as the character of the residues involved in dimer formation and the overall nature of their interactions are clearly compatible with a crystallisation-induced contact, but not with a physiologic dimer. Furthermore, boophilin behaves as a monomer in gel filtration chromatography (data not shown). The two complexes are virtually identical (r.m.s.d. of 0.58 Å for 419 equivalent Cz atoms), thus only complex A (thrombin chains A and B and boophilin chain E) will be described.

By contrast to the major rearrangements of loops surrounding the active site centre observed in the (E192Q)thrombin-BPTI complex, the structure of thrombin is virtually unaltered upon boophilin binding. In comparison to the unliganded molecule (e.g., free z-thrombin from PDB entry 1MKX) 279 Cz atoms of the proteinase can be aligned with a r.m.s.d. of only 0.59 Å.
Significantly different arrangements of boophilin and ornithodorin in their complexes with thrombin

The inter-domain linker is three residues longer in boophilin than in ornithodorin. Nevertheless, it runs closer to the proteinase surface in thrombin bound to the former inhibitor (Figure 7A). This linker peptide fits tighter to thrombin’s surface features and engages in new interactions with thrombin not seen in the thrombin-ornithodorin complex, further contributing to inhibitor binding. The most relevant of these are hydrophobic/aromatic contacts of $^{B}F80$ with $^{T}F34$, as well as a water-mediated hydrogen bond between the carboxylic acid of $^{B}E81$ and the main chain nitrogen of $^{T}Y76$ (Figure 7B). (We use superscripts “B” and “T” before residue names to denote boophilin and thrombin residues, respectively).

Unexpectedly, the two Kunitz domains of boophilin not only diverge largely in three-dimensional structure from ornithodorin, but also occupy significantly different positions relative to the proteinase moiety, when compared to the thrombin-ornithodorin complex (Figure 7A). In particular, the N-terminal boophilin moiety rotates about nine degrees and is shifted $\approx 6\,\text{Å}$ to the “left” in the standard orientation depicted in this figure. As for the C-terminal module, it is in boophilin rotated by approximately six degrees and translated by $\approx 3\,\text{Å}$ “upwards”. These differences in quaternary arrangement of Kunitz modules relative to the serine proteinase moiety result in large differences when compared to the thrombin-ornithodorin complex, as we discuss below.

Water-mediated interactions dominate at the interface between thrombin exosite I and boophilin

The C-terminal domain of boophilin engages in both hydrophobic and electrostatic interactions with thrombin’s exosite I. In contrast to the thrombin-ornithodorin complex, however, where direct interactions between side chains of the proteinase and the inhibitor dominate at this interface, in the boophilin complex the equivalent residues are mostly involved in water-mediated interactions (Figure 7B). In addition, the guanidinium group of $^{T}R77A$ donates hydrogen bonds to the hydroxyl group of $^{B}Y129$ and the carbonyl oxygen of $^{B}D126$. Further direct hydrogen bonds are formed between the carboxylate of $^{B}E134$ and the hydroxyl group of $^{T}Y76$. These polar contacts are strengthened by hydrophobic interactions between the side chains of $^{B}L137$ and a pocket formed by proteinase residues $^{T}L65$, $^{T}R67$ and $^{T}I82$, as well as between $^{B}V138$ and $^{T}Y76$.

We note that the exosite I-contacting face of boophilin, although displaying an overall negative electrostatic potential, harbours a smaller cluster of acidic residues than ornithodorin,
which are located closer to the interdomain linker. Unexpectedly, none of the three salt bridges between glutamates in ornithodorin and the proteinase moiety are observed in the complex with boophilin, even though these acidic residues are conserved or conservatively replaced. Likewise, of the five residues involved in hydrophobic contacts in ornithodorin, one (I117) has no equivalent in boophilin, while the structural equivalents of two others (Y129 for F103 and E133 for V107) have an increased polar character and are no longer involved in van-der-Waals interactions (Figure 7B).

Although the total buried area upon thrombin complexation by boophilin is equivalent to that of the thrombin-ornithodorin complex (~1,800 Å²), the decrease in the number and strength of the proteinase-inhibitor interactions offers an explanation for the lower $K_i$ determined for boophilin (see Discussion).

It is noteworthy that a good fraction of boophilin’s negative charges cluster in the solvent exposed side of the molecule, pointing away from the basic exosite I of thrombin (Supplementary Figure S2). This suggests that the C-terminal domain serves a multiple role, sterically blocking substrate access, masking the overall positive charge of the exosite, and creating a new negatively-charged surface that can repel approaching thrombin substrates. These combined effects would thus counteract competitive displacement of the inhibitor by physiological substrates, which require previous exosite I binding for processing.

The N-terminal residues of boophilin occupy the active site cleft in a parallel manner

The experimental electron-density map (Figure 8A) unambiguously shows that the N-terminal residues of boophilin bind across thrombin’s active site cleft in a parallel, hirudin-like manner [16]. In the three-dimensional structure of the thrombin-ornithodorin complex, it was observed that the introduction of a non-natural
serine residue at the N-terminus of the inhibitor results in an unfavourable chemical environment around the side chain of TE192. By contrast, the present structure shows that this carboxylate group is stabilised by hydrogen-bonding interactions with the side chain of the natural N-terminal residue of mature boophilin, BQ16 (see Figure 8B for a schematic representation of major thrombin-boophilin interactions). Inspection of the electron density maps suggests that the latter side chain displays a somewhat increased flexibility, which correlates well with its above-average B-factor. The main chain of this N-terminal residue is positioned in the S2 pocket of thrombin, stabilised by a direct hydrogen bond between its carbonyl oxygen and the guanidinium group of BR22.

The most relevant feature of active site interactions is the occupancy of thrombin’s S1 subsite by the ideally suited side chain of BR17. The guanidinium group of this boophilin residue forms two hydrogen bonds with the carboxyl group of TD189 (3.4 and 3.6 Å) at the bottom of the S1 pocket (Figure 8A). Two additional hydrogen bonds are formed between the terminal group of BR17 and the main chain carbonyls of TG219 (direct bond) and TF227 (water-mediated). This feature also distinguishes boophilin from ornithodorin, which lacks an equivalent residue at this position.

The following boophilin residue, BN18, interacts through its side chain with the main chain nitrogen atom of G219 and the carboxyl oxygen of E146, the latter via a solvent molecule. Finally, G19 is hydrogen-bonded to the carbonyl group of G216 (Figure 8B), thus partially mimicking the interaction of a ligand occupying the S1 pocket of the proteinase. This side chain accommodates the side chain of R22, which forms a direct hydrogen bond with the hydroxyl group of Y60A. The only other major contact between the proteinase moiety and the N-terminal domain of boophilin is the parallel stacking of the side-chain of F39 with those of P60C and W60D.

Discussion

Tick-derived anti-hemostatics

We report the isolation and cloning of a two-domain Kunitz inhibitor from the cattle tick Boophilus microplus, which we have termed boophilin. Further, we characterise boophilin’s inhibitory mechanism by solving the three-dimensional structure of its complex with bovine thrombin. Together with our identification of carrapatin, a single domain Kunitz-type inhibitor in engorged ticks (Swiss-Prot accession number P81162), as well as with previous reports of inhibitors isolated from eggs and larvae of this parasite [28–34,40], evidence is emerging that B. microplus utilizes a whole array of both single- and multi-domain Kunitz inhibitors during its whole life cycle. Recently, several salivary proteins that contain single or tandem Kunitz repeats have also been identified in the hard ticks Ixodes scapularis [41] and I. pacificus [42], indicating that inhibitors from this family might play a general role as antihemostatic factors. One of these proteins, the double-headed inhibitor, ixolaris, was shown to inhibit the tissue factor-FVIIa complex in a FX(a)-dependent manner by binding to exosite I in FXa, in line with its homology to TFPI [43,44].
Recently, another two-domain Kunitz inhibitor with anti-thrombin activity, amblin from the hard tick Anghydomus hebraeum, has been described [37]. Notably, amblin differs from boophilin due to (i) a much shorter interdomain linker; (ii) a relatively long, highly basic C-terminal extension; (iii) the presence of two additional cysteine residues located in this extension and in the C-terminal Kunitz domain, respectively, which might form an extra disulfide bond; and (iv) non-conservative replacements of several acidic residues of the C-terminal domain (Figure 5). We have generated a three-dimensional model of amblin on the basis of its closest relative of known structure, bikunin [45]. The high pH of amblin is reflected in extended areas of basic potential on both Kunitz domains (Supplementary Figure S2). Thus, both strong electrostatic repulsion and inappropriate quaternary structure would make extremely unlikely that amblin interacts with any of the positively charged exosites on the thrombin moiety. The lack of anti-trypsin activity by amblin is also surprising in view of the conservation of “canonical” reactive-site loops, and because our homology model suggests that the reactive-site loop of the N-terminal Kunitz domain remains accessible to target proteinases. These observations require a thorough investigation of amblin’s inhibitory potential.

Boophilin is a canonical Kunitz-type inhibitor

Inhibition of the key procoagulant enzyme, thrombin, by canonically binding Kunitz-type inhibitors is unfavourable due to the presence of a prominent insertion loop at position 60, and of a glutamate at position 192. These obstacles are circumvented in the double-headed Kunitz inhibitor from O. moubata, ornithodorin, as only its N-terminal peptide inserts into the active-site cleft of the proteinase [25]. A highly similar thrombin inhibitor, savignin, has been isolated from the salivary glands of O. savignyi [46,47], and would be expected to use the same non-canonical mechanism for thrombin inhibition. On the other hand, the close resemblance of boophilin to canonical Kunitz domains and the presence of an optimal P1 residue in its N-terminal domain (K31) suggested that boophilin could inhibit thrombin in a canonical manner, i.e. by occupying the active-site cleft of the proteinase with the reactive-site loop of this module. This would require a considerable repositioning of the N-terminal Kunitz module compared to the thrombin-ornithodorin complex, which is nevertheless possible given that the two domains are connected by a long, flexible linker. Canonical binding would in turn require that initial binding of the C-terminal domain to exosite I provided enough energy to drive insertion of the N-terminal reactive site loop past the energetic barrier that thrombin’s 60-loop represents.

Boophilin inhibits thrombin in a non-canonical manner, despite possessing a canonical reactive-site loop

However, structural and functional evidence presented here indicates that boophilin also adopts the non-canonical mechanism for thrombin inhibition, in spite of possessing a regular BPTI-like domain. We conclude that binding of the acidic C-terminal Kunitz domains to exosite I does not induce major rearrangements of loops surrounding thrombin’s active-site cleft. This is in line with our previous observations of essentially unmodified active sites in the crystal structures of thrombin-triabin [15] and thrombin-thrombomodulin [24] complexes. A recent crystal structure of murine thrombin bound to an acidic peptide from PAR3 via exosite I revealed some displacements of 60-loop residues, in particular W60D [48]. However, analysis of the thrombin-PAR3 complex (PDB entry 2PUP) indicates that these rearrangements are either induced or stabilised by artificial interactions with a neighbouring crystal molecule. In conclusion, it would seem that interactions with exosite I neither induce rearrangements of thrombin structure, in particular of the 60-loop, nor are required for inhibitors or substrates to approach the proteinase catalytic machinery. Exosite-mediated interactions are nevertheless strictly required for positioning unfavourable substrates (protein C, thrombin-activatable fibrinolysis inhibitor/TAFI)/inhibitors (Kunitz) at a proper distance and orientation to gain access to the active site of thrombin.

In spite of similar domain organisations and the presence of highly acidic C-terminal domains, ornithodorin/savignin are much tighter thrombin inhibitors ($K_i = 1.0 \times 10^{-12}$ M or $4.9 \times 10^{-12}$ M) than boophilin ($K_i = 1.8 \times 10^{-9}$ M). Further, whereas ornithodorin and savignin do not cross-react with other serine proteinases, boophilin is a potent inhibitor of trypsin and of the fibrinolytic enzyme, plasmin. It is tempting to speculate that the less efficient thrombin inhibitor, boophilin, confers an evolutionary advantage to B. microplus by controlling opposing mechanisms of hemostatic balance. Another interesting possibility is discussed...
below. Of note, a thrombin inhibitor with an apparent M, ~60 kDa, BrnAP, has been isolated from the saliva of *B. microplus* [49]. In contrast to boophilin, BrnAP does not inhibit plasmin or trypsin. Finally, a small (M, = 1,770 Da) exosite-binding thrombin inhibitor, microphilin, has been identified in the tick saliva [50]. It is thus conceivable that the three inhibitors cooperate to inhibit thrombin activity during feeding and digestion.

It is also worth mentioning that immunization of cows with salivary gland preparations from *B. microplus* reduced babesiosis [51], pointing to the immunological potential of proteins secreted by this parasite. Of note, the sequence containing the reactive-site loop in boophilin N-terminal domain is predicted to be the major antigenic determinant of the protein. Given that thrombin inhibitors are critical for hematophagous animals during feeding and digestion, boophilin would appear as a promising candidate for the development of recombinant vaccines.

The close relatedness of boophilin to BPTI-like Kunitz inhibitors (Figures 3 and 6) explains its ability to potently inhibit trypsin and plasmin. In particular the N-terminal domain possesses all the necessary features for canonical inhibition, including a lysine side chain (K31) ideally suited for occupying the S, pocket of trypsin-like serine proteinases with a serine at position 190 [7,52,53]. Further, the I29 side chain is preferred at the P, position in Kunitz-type inhibitors [53]. Finally, the conserved alanine residue at position P, is also essential for formation of stable complexes with trypsin and plasmin [54]. For several other serine proteinases tested, the presence of A190 at the bottom of the S, specificity pocket, instead of a serine in e.g. trypsin and plasmin, increases the size of this pocket and eliminates a potential hydrogen-bond partner for a lysine side chain [55]. In these cases, Kunitz inhibitors with an arginine at position P, are strongly preferred over those with a P, lysine [52,53], thus explaining at least partially the poor or absent effect of boophilin on FXa, FXIIa, uPA and t-PA. Finally, in the trypstat tetramer the active sites of all four monomers are arranged facing an internal pore, and are therefore inaccessible to most substrates and inhibitors [56]. Kunitz inhibitors are too bulky to enter the trypstat channel, explaining the inactivity of boophilin towards trypstat.

**Boophilin is a bi-functional serine proteinase inhibitor**

As mentioned above, boophilin possesses a rather high $k_i$ for thrombin inhibition when compared to the two other thrombin inhibitors known or predicted to bind in a non-canonical manner, ornithodorin [25] and savignin [46,47]. This observation raises the interesting possibility that boophilin could have evolved to target not only circulating thrombin, but to block in addition the membrane-bound activation intermediate, mezithrombin (MzT). The crystal structure of a MzT variant that lacks the membrane-binding fragment 1, reveals formed exosite I and active centre, while exosite II is covered by the kringle 2 domain of the intermediate form [57]. Therefore boophilin could dock to this form essentially as seen in the current crystal structure (see Figure 9A for a representation of the modelled MzT-boophilin complex).

Our current data also indicate that thrombin-bound boophilin retains the capability to interact with additional serine proteinases, as demonstrated by formation of a stable ternary complex with trypsin (Figure 4), and the reactive-site loop of the N-terminal boophilin domain would remain fully accessible in the MzT-boophilin complex (compare Figures 3 and 9A). These observations immediately suggest that a proximal, membrane-bound proteinase could easily dock to this complex. Coagulation FXa would appear as the most attractive candidate, because of its proximity to MzT during prothrombin activation by the prothrombinase complex (FVa-FXa). To verify that MzT-boophilin-FXa ternary complex formation is feasible without steric clashes, we overlaid the structurally and functionally related second Kunitz domain of TFPI (refer to Figure 6A) bound to trypsin [6] onto the N-terminal boophilin domain. Next, we superposed the FXa crystallographic model [58] onto the trypsin moiety. This revealed that the serine proteinase moieties of FXa and mezithrombin could be cross-linked by the N-terminal domain of a boophilin molecule (Figure 9B). Furthermore, the N-terminal domains of both proteinases would not interfere with ternary complex formation. In this manner, boophilin could efficiently inhibit thrombin generation by prothrombinase. A schematic representation of this putative, membrane-bound complex is shown in Figure 9C. We stress, however, that free boophilin only marginally inhibits FXa in *vitro* (Table 1), and thus formation of the ternary complex would rely on the proper orientation of both membrane-bound serine proteinases, and would probably require additional boophilin-FVa interactions. In support of our hypothesis, we note that both endogenous (TFPI) [4] and tick-derived multidomain Kunitz inhibitors ixolaris [43,44] and penthalarin [59] employ this dual strategy for inhibiting the related FVIIa-TF-FXa complex. Of particular note, TFPI inhibits FVIIa through its first Kunitz domain, but only after the second Kunitz moiety has bound FXa [4,60]. Future investigations should test the validity of this model, or identify other targets for thrombin-bound boophilin in *vivo*.

Finally, we notice that the current structure has implications for modelling procedures, in particular for macromolecular docking. Current docking algorithms have only had limited success rates, even when the structures of isolated modules were known [61]. Our structure of the thrombin-boophilin complex and its comparison with the related thrombin-ornithodorin heterodimer reveals that caution must be exercised even when a highly homologous template for the expected complex does exist. Indeed, these two inhibitors share a common binding mode (blockade of basic exosite I of the proteinase and parallel alignment of N-terminal residues across the active-site cleft). However, the details of interacting residues (e.g., direct vs. water-mediated contacts at exosite I, occupancy or not of the major S, subsite on thrombin) vary considerably in their complexes with the proteinase. Concomitantly, and perhaps more striking from the viewpoint of macromolecular docking, the two pairs of inhibitor domains adopt markedly different orientations relative to the cognate thrombin molecule.

**Materials and Methods**

**Animals**

Ticks were obtained from Drs. D.H. Aguirre and A.B. Gaido, Instituto Nacional de Tecnología Agropecuaria, Estación Experimental Agropecuaria Salta (Argentina).

**Reagents**

Bovine z-thrombin was isolated from fresh ox blood as described previously [62]. Other serine proteinases were purchased from the suppliers listed below: bovine FXa, Diagnostic Reagents Ltd. (Oxon, England); human z-FXIIa and plasma kallikrein, Kordia Laboratory Supplies (Leiden, Netherlands); recombinant human FXIIa (NovoSeven®, Novo Nordisk (Denmark); human plasmin, Behringwerke GmbH (Marburg, Germany); recombinant trypstat, Promega (Madison, USA); recombinant sc-IPA, Boehringer Mannheim (Mannheim, Germany); bovine pancreatic trypsin, Serva Feinbiochemika (Heidelberg, Germany); and urokinase (uPA), Ribopharm GmbH (Haan, Germany). All chromogenic substrates used were obtained from PentaPharm AG (Basel, Switzerland), except for Chromozym® TH (Tos-Gly-Pro-Arg-p-nitroanilide), purchased from Boehringer Mannheim, which also provided APTT reagent and endoproteinases Lys-C and Asp-N. Dade® Thromboplastin-IS was purchased from Dade Behring Marburg (Marburg, Germany).
Figure 9. Predicted boophilin interactions with meizothrombin and FXa. (A) Model of the three-dimensional meizothrombin-boophilin complex, generated by overlaying the coordinates of bovine meizothrombin (blue except for the Kringle 2 domain, in green; 1A0H) [57] onto the thrombin moiety of the current complex with boophilin. Residues R17 and K31 are shown as space-filling models and labelled. (B) Model of the putative MzT-boophilin-FXa ternary complex. FXa (yellow, except for domain EGF2, in olive; 1FJS) [58] was docked onto the complex displayed in (A) by juxtaposition onto a trypsin-APP complex (1TFX) [6] that had been previously overlaid onto the N-terminal domain of boophilin (red). Absence of any steric clashes shows that formation of such a ternary complex in vivo would be feasible. The N- and C-terminal domains of boophilin (Boophilin (N) and Boophilin (C), respectively), the serine proteinase and Kringle 2 domains of meizothrombin (SP (MzT) and Kringle 2, respectively), and the serine proteinase and EGF-2 domains of factor Xa (SP (FXa) and EGF-2, respectively) are labelled. (C) Schematic representation of a possible mechanism for boophilin inhibition of the membrane-bound prothrombinase complex. Both FXa and meizothrombin interact with the membrane surface (dark red) via their respective Gla domains (light green). The multi-domain organisation of FVa (violet) and its contacts with FXa/MzT have been omitted for simplicity. The N-terminal domain of boophilin (red) could bridge the catalytic domains of meizothrombin (dark green) and FXa (blue) while bound to the cofactor.

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All other chemicals, of the highest purity grade available, were purchased from Merck (Darmstadt, Germany), except where noted.

Isolation of native boophilin

One-hundred fifty grams of frozen engorged ticks were homogenised in 1 l buffer A (20 mM Tris-HCl, pH 8.0, 1 mM CaCl$_2$, 1 mM benzamidine). After centrifugation at 10,000 × g for 10 minutes, the filtered (5-μm pore) supernatant was applied to a Q-Sepharose (Pharmacia, Freiburg, Germany) column (200 × 250 mm) previously equilibrated with 20 mM Tris-HCl, pH 8.0 (buffer B). The column was extensively washed with buffer B and bound proteins were eluted with a linear NaCl gradient (from 0 to 1 M in buffer B).

A thrombin affinity column was prepared by coupling 10 mg of bovine z-thrombin to 2 g of CNBr-activated Sepharose 4B (Pharmacia), according to the manufacturer’s guidelines. Ion-exchange chromatography fractions with thrombin inhibitory activity (determined as described below) were pooled, diluted with two volumes of 20 mM sodium phosphate, pH 7.4 (buffer C) and applied to the thrombin-Sepharose column previously equilibrated in buffer C. The column was extensively washed with buffer C and subsequently with buffer C containing 500 mM NaCl and with double distilled water, before elution of specifically bound proteins with 20 mM glycine-HCl, pH 2.5. The pH of the eluate was immediately adjusted to 8.0 with 0.1 volumes 1 M Tris-HCl, pH 8.0 before lyophilising the sample.

Activity assays

Thrombin inhibitory activity was followed during boophilin purification by measuring the residual amidolytic activity of the proteinase towards Chromozym® TH. Bovine thrombin (900 μl, 15 nM) in assay buffer (50 mM Tris-HCl, pH 7.8, 0.1 M NaCl, 0.05% PEG 6,000) was incubated with 50 μl protein samples or with the same volume of assay buffer for 2 minutes at 37°C. Substrate (50 μl, 1.5 mM) was then added, and absorbance of the reaction mixture at 405 nm was monitored for 2 minutes.

Inhibition of blood clotting by boophilin

To determine thrombin time, 100 μl citrated human plasma were mixed with 50 μl 0.154 M NaCl or inhibitor solution and equilibrated at 37°C. Bovine thrombin (50 μl; 5 IU/ml) was then added and the time to clot was measured. Prothrombin time was determined by mixing 50 μl of Dade® Thromboplastin-IS with 50 μl of either 23 mM CaCl$_2$ or the same solution containing the sample to be assayed. After equilibration at 37°C, 50 μl human citrated plasma were added, and the coagulation time measured. Activated partial thromboplastin time was determined both with and without preincubation of the inhibitor solution (40 μl) with 50 μl citrated human plasma and 50 μl APTT reagent for 3 minutes at 37°C. Ten μl of a 125 mM CaCl$_2$ solution were finally added and the coagulation time was measured. Clotting times were determined in duplicate.

Inhibition of other serine proteinases

The amidolytic activity of each proteinase towards a specific chromogenic substrate was assayed at room temperature, by measuring the variation of light absorbance at 405 nm in the assay mixture. Briefly, 200 μl of assay buffer (50 mM Tris-HCl, pH 7.4–8.4, 154 mM NaCl) or of inhibitor were mixed with 50 μl of the enzyme solution and dispensed in 96-well microtiter plates. After five minutes pre-incubation, 25 μl of the chromogenic substrate solution were added and the reaction was allowed to proceed for an enzyme-dependent period (2–6 minutes). Reactions were stopped by adding 25 μl 50% (v/v) acetic acid. The enzymes tested are given in the legend to Table 1, along with the chromogenic substrates used in each case.

For determination of the second-order rate constants of trypsin/ plasmin inhibition, 200 μl of inhibitor solution were mixed with 25 μl substrate (Chromozym® TH or Chromozym® PLg, respectively; final concentration: 0.2 mM). The final concentration of recombinant boophilin was varied between 0.1 and 0.65 μM (trypsin), or between 0.04 and 0.4 μM (plasmin). The reaction was started by addition of 25 μl enzyme solution (final concentrations: 0.25 ng/ml and 12.5 μg/ml, respectively), and the absorption at 403 nm was measured over 45 minutes at 37°C. The pseudo-first order rate of inactivation for each inhibitor concentration (k$_{inh}$) was determined from the progression curve according to the equation [P] = A+Bt−Cexp(−k$_{inh}$t). Second-order rate constants were calculated from the equation k$_{obs}$ = (1+$S_0$)/K$_{inh}$/b, with S$_0$ = 0.2 mM, K$_{inh}$ = 0.0117 mM for Chromozym® TH or 0.286 mM for Chromozym® PLg; the slope b was computed from the regression line 1/k$_{inh}$ versus 1/[I].

Electrophoretic mobility shift assays

Five-hundred ng human thrombin or bovine trypsin in 25 mM Tris-HCl pH 8.0, 190 mM glycine, were mixed with increasing concentrations of boophilin and incubated at room temperature for 15 min before adding glycerol to 5% final concentration. In other experiments, thrombin-boophilin complexes were similarly titrated with increasing concentrations of trypsin. Samples were resolved at 4°C in Tris-glycine-polyacrylamide gels and silver-stained.

Protein sequencing

Lyophilised protein samples were dissolved in 0.1 M Tris-HCl, pH 8.0, 6 M guanidinium hydrochloride, 5% (v/v) β-mercaptoethanol and incubated for 16 hours at room temperature to reduce disulfide bonds. The free thiol groups were derivatised with 5% (v/v) 4-vinylpyridine; samples were incubated for further 90 minutes at room temperature, acidified with formic acid, and finally desalted by RP-HPLC. The S-$β$-pyridylethylated samples were digested for 10 hours at 37°C with either Lys-C (in 25 mM Tris-HCl, pH 8.5, 1 mM EDTA) or Asp-N (in 50 mM sodium phosphate, pH 8.0). Reactions were stopped by adjusting the pH of the samples to 2.0 with formic acid. For CNBr-cleavage, S-$β$-pyridylethylated boophilin was dissolved in 70% (w/v) formic acid containing 10% (w/v) CNBr and incubated in the dark at room temperature for 14 h. Peptides were separated by RP-HPLC and subjected to automated sequencing. The molecular mass of the unmodified protein was determined by MALDI-MS.

Construction and screening of the B. microplus cDNA library

Total RNA was isolated from 1 g of unfed ticks (for cDNA library construction) or from 10 g of engorged adults (for PCR amplification) and enriched in poly(A)$^+$ RNA by oligo(T)-cellulose affinity chromatography. cDNA was synthesised from 1 μg of poly(A)$^+$ RNA with random hexanucleotides as primers, and a cDNA library was constructed in λ Uni-ZAP XR (Stratagene, Amsterdam, Netherlands), following the manufacturer’s instructions.

An internal fragment of boophilin cDNA was amplified by PCR using degenerate oligonucleotides (Supplementary Table S2), cloned into the BamHI site of pBluescript KS (Stratagene, Amsterdam, Netherlands), and sequenced. A fragment of similar size was amplified using aliquots of the cDNA library phage stock as template, to verify the presence of boophilin cDNA in the library. Positive clones, identified using the 25-P-dATP-labelled boophilin
cDNA fragment as probe, were isolated and converted into double stranded pBluescript phagemids by in vitro excision, and further characterised by automated DNA sequencing of both strands.

Heterologous expression of mature boophilin

Mature boophilin (variant H2) was cloned into the bi-cistronic expression vector pRBI-DsbsC [63] by overlap-extension PCR. Briefly, the OmpA signal-coding region of the vector and the fragment of boophilin cDNA coding for the mature inhibitor were amplified independently, using specific primers (Supplementary Table S2). Aliquots of both PCR products were then mixed and amplified using the previously used 5’ primer for the OmpA fragment and the 3’ primer for boophilin. The amplified fragment, coding for an OmpA signal–mature boophilin fusion protein was cloned into the Xba I and Hind III sites of the expression plasmid. *Escherichia coli* DH5α cells transformed with the expression construct were grown in LB with 2 g/l glucose and 100 mg/l ampicillin to an optical density of 0.7, before induction with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). At induction time, 2 g/l glucose, 100 mg/l ampicillin, and 5 mM N-acetyl-cysteine were added. Cells were harvested 36 hours post-induction and periplasmic proteins were released via cold osmotic shock. The resulting solution was immediately applied to an 80×50 mm Q-Sepharose FF column pre-equilibrated with buffer D (20 mM Tris-HCl, pH 8.0). The column was extensively washed with buffer D, and bound proteins eluted with a linear salt gradient (from 0 to 0.5 M NaCl in buffer D). Fractions displaying anti-thrombin activity were pooled, diluted with buffer D and loaded onto a ResourceQ column (Pharmacia, Freiburg, Germany) pre-equilibrated with the same buffer. The column was extensively washed with buffer D and bound proteins were eluted with a linear salt gradient (0–0.4 M NaCl in buffer D). Samples with anti-thrombin activity eluted as a single peak. Correct processing of the recombinant inhibitor was verified by MALDI-MS and N-terminal sequencing.

Complex preparation and crystallisation

The macromolecular complex was prepared by mixing bovine α-thrombin with a molar excess of purified recombinant boophilin. The mixture was incubated on ice for two hours, and the thrombin-boophilin complex was separated from excess inhibitor by size exclusion chromatography on a Superdex 75 10/30 column (Amersham Pharmacia), using 0.01 M MES pH 6.5, 0.1 M NaCl as the mobile phase. Fractions were collected and analysed by SDS-PAGE. Those containing the thrombin-boophilin complex were pooled and concentrated on a 10-kDa cut-off centrifugal concentrator (Vivascience).

Single crystals of the macromolecular complex were obtained by vapour diffusion at 279 K from 4-m KH2PO4, 18% (w/v) PEG 8,000). Crystals belong to the orthorhombic space group P212121, with unit cell constants a = 92.5 Å, b = 104.2 Å, c = 129.1 Å, and contain two complexes per asymmetric unit (solvent content 60.6%). The crystals were transferred to 1.5 M mother liquor containing 15% glycerol as cryoprotectant and cryo-cooled in liquid nitrogen.

Data collection, processing and refinement

Diffraction data to 2.35 Å were collected from a single cryo-cooled (100 K) crystal on a MAR CCD detector at ESRF beamline ID14-EH3. Data were processed with MOSFLM [64], scaled with SCALA [65], and reduced with tools from the CCP4 package [65]. Data collection and refinement statistics are summarised in Table 2.

| Table 2. Statistics of data collection and refinement |
|------------------------------------------------------|
| **Crystallographic analysis**                        |
| Resolution range (Å) (overall/outer shell)           | 54.9–2.35/2.48–2.35 |
| Space group                                          | P212121              |
| Unit cell dimensions (Å)                             | a = 92.5; b = 104.2; c = 129.2 |
| Number of observations (total/unique)               | 192,392/51,554       |
| Multiplicity (overall/outer shell)                   | 3/7/3/7              |
| Rmerge (%) (overall/outer shell)                     | 6.4/29.0             |
| Completeness (%) (overall/outer shell)              | 98.0/97.4            |
| l(cm) (overall/outer shell)                          | 9.5/2.6              |
| Mathews coefficient (Å³ Da⁻¹)                        | 3.1                  |
| Solvent content (%)                                  | 60.6                 |
| **Structure refinement**                             |
| Rfactor/free Rfactor (%)                             | 19.0/23.0            |
| N° of unique reflections (working/test set)          | 48,886/2,627         |
| Water molecules                                     | 453                  |
| Ions                                                 | 6 (4 PO₄²⁻, 2 Na⁺)   |
| Total number of atoms                                | 7,261                |
| Number of protein atoms                              | 6,771                |
| Average overall B-factor (Å²)                        | 31.2                 |
| Average protein B-factor (Å²)                        | 30.8                 |
| Average main-chain B-factor (Å²)                     | 30.5                 |
| Average side-chain B-factor (Å²)                     | 31.2                 |
| Average water B-factor (Å²)                          | 33.1                 |
| Average ion B-factor (Å²)                            | 62.4 (PO₄²⁻)/32.5 (Na⁺) |
| r.m.s.d. bonded Bs (Å)                               | 0.751                |
| r.m.s.d. bond lengths (Å)                            | 0.008                |
| r.m.s.d. bond angles (°)                             | 1.137                |
| **Ramachandran plot statistics**                     |
| Residues in allowed regions                         | 717 (99.9%)          |
| Residues in generously allowed regions               | 1 (0.1%)             |
| Residues in disallowed regions                       | 0 (0%)               |
| **Estimated coordinate error**                       |
| E.s.d. from Luzzati plot (Å)                         | 0.30                 |
| DPf (Å)                                              | 0.20                 |

The structure was solved by molecular replacement using AMoRe [66] with data in the 10–4.0 Å resolution range. Two proteinase molecules were located using the coordinates of unliganded bovine α-thrombin from PDB entry 1MKX [67] as search model. All attempts to locate the Kunitz domains of boophilin by molecular replacement, using truncated models of either BPTI (BPI) [68] or ornithodrin (1TOC) [23] were unsuccessful.

The initial electron density maps, computed after rigid body refinement of the proteinase molecules with CNS v. 1.1 [69],
displayed additional density for most of the inhibitor molecules. Two cycles of manual rebuilding of the proteasome models on a graphic workstation using TurboFRODO (Bio-Graphics, France), followed by positional refinement with CNS resulted in significant improvement of the electron density maps. This allowed manual docking of a truncated model of BPTI close to thrombin exosite I for each of the complexes. Further, crystallographic refinement alternated with a manual model building until complete interpretation of the electron density maps. The final isotropically refined model obtained with CNS was further refined with REFMAC-5 [70] using TLS displacement parameters.

The final model comprises residues 1–F11P to R15 and 1–I16 to 1–D243 of one thrombin molecule (chains A and B, respectively) and residues 1–P11N to 1–E14L and 1–I16 to 1–D243 of the other protease moiety (chains C and D, respectively). Both boophilin molecules (chains E and F) were modelled from Q616 to M142. The side chains of 1–E14L (chain A), 1–K236 (chain B), 1–K11, 1–K14D (chain C), 1–K87, 1–R95, 1–K97, 1–E113 (chain D), 1–P75, 1–E110 (chain E), and 1–K86 (chain F) are not well defined in the electron density maps; we have assigned an occupancy value of zero to these side chains. The model comprises also three phosphate and two sodium ions, 453 water molecules and an N-acetyl-glucosamine sugar moiety attached to N60G in chain D.

Modelling and other computational methods

Sequence similarity searches were performed with the program FastA from the Wisconsin package (version 9.0, Genetics Computer Group, Madison, Wisconsin), and alignments were prepared with ALSCRIPT [71]. Major antigenic peptides were predicted with the Kolaskar & Tongaonkar method [http://bio.dfci.harvard.edu/Tools/antigenic.pl]. Models of the thrombin-boophilin complex were built based on the co-ordinates of 1E192Q thrombin-BPTI [18TH] [23] and thrombin-ornithodorin (1TOC) [25] complexes, and were subjected to energy minimization with CNS. The amblin model was built by applying homology-based methods as implemented in the automatic SWISS-MODEL server [http://swissmodel.expasy.org] [72], using the structure of the Kunitz inhibitor bikunin as template [45]. Structure representations were generated with PyMOL (http://www.pymol.org) or SETOR [73].

Accession codes

The complete nucleotide sequences of both boophilin isoforms have been deposited with the EMBL database under accession numbers AJ304446 and AJ304447, respectively. Coordinates for the bovine γ-thrombin-boophilin complex have been deposited with Protein Data Bank, accession number 2ODY.

Supporting Information

**Figure S1** Native boophilin is a small disulfide-linked, acidic thrombin inhibitor. Mass spectrum of affinity-purified native boophilin obtained using MALDI-TOF. Notice the position of the myoglobin marker (both M+H and M+2H* peaks). The inset shows the electrophoretic analysis of purified boophilin separated in a 15% SDS-polyacrylamide gel, stained with Coomassie Brilliant Blue. Lane 1, non-reduced boophilin sample; lane 2, molecular mass marker; lane 3, reduced boophilin; molecular mass markers are given to the right.

**Figure S2** Two-domain Kunitz inhibitors of thrombin display different surface potentials. Electrostatic surface potential (red, negative; blue, positive) representation of (A) boophilin, (B) ornithodorin, and (C) amblin. For image pairs in panels (A) and (B) the left image corresponds to the exposed side of the molecule when bound to the protease, and the right image results from a 180° rotation around the y-axis, to expose the thrombin-binding surfaces to the viewer. The model of amblin is represented with its N-terminal domain in a similar orientation to those of the other inhibitors. Selected topologically equivalent residues in boophilin and ornithodorin are labelled.

**Table S1** Amino acid sequences of peptides derived from purified native boophilin. Reduced and S-φ-pyridylethylated samples were digested with endoproteinases Lys-C or Asp-N, or chemically hydrolysed with CNBr. The resulting fragments were separated by RP-HPLC and major peaks were sequenced; peptides obtained by either procedure are numbered according to their elution order in the corresponding chromatograms. Positions at which two different amino acid residues have been obtained are underlined; they indicate the existence of at least two boophilin isoforms. Overlapping peptides CNBr-Asp-N4-(Lys-C5/ Lys-C6)-Asp-N2-Lys-C13-Asp-N7 define a single sequence of 77 residues: FSYGGC'GNENNFAETEDC'QKAC6GAPERV(N/ S)DFE/S/GADFKTGC'EPAADSGS/GC'AGQLERFYVNRS- GEC/G ETFVYGGC/S/G/G. Based on the homology to BPTI, peptide Lys-C11 appears to correspond to the segment between the second and the third cysteine residue of the N-terminal domain, i.e. preceding the assembled sequence, while Asp-N8 could be assigned to the region immediately following the C-terminal residue in the assembled sequence.

**Table S2** Oligonucleotides used for boophilin cloning. The BamHI (primers Lys-C15F and Lys-C15R) and Hind III sites (BamF and HindIII) introduced for cloning purposes are underlined. R, Y, and N, represent equimolar amounts of A and G, C and T, and of all four bases, respectively. The triplet coding for Q16 in primer BohF and the termination codon in Boh-Rev are given in bold letters.

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**Author Contributions**

Conceived and designed the experiments: PP SM PF. Performed the experiments: PP SM CA BC RM JS PF. Analyzed the data: PP SM PF. Contributed reagents/materials/analysis tools: PP SM TF RM JS PF. Wrote the paper: PP SM PF.
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