A Carboxypeptidase Inhibitor from the Tick Rhipicephalus bursa

ISOLATION, cDNA CLONING, RECOMBINANT EXPRESSION, AND CHARACTERIZATION*

Received for publication, September 27, 2004, and in revised form, November 2, 2004
Published, JBC Papers in Press, November 23, 2004, DOI 10.1074/jbc.M411086200

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A novel proteinaceous metallo-carboxypeptidase inhibitor, named tick carboxypeptidase inhibitor (TCI), was isolated from the ixodid tick Rhipicephalus bursa and N-terminally sequenced. The complete cDNA encoding this protein was cloned from tick mRNA by reverse transcription-PCR and rapid amplification of cDNA ends techniques. The full-length TCI cDNA contains an open reading frame coding for a precursor protein of 97 amino acid residues that consists of a predicted signal peptide of 22 residues and of mature TCI, a 75-residue cysteine-rich protein (12 Cys). The deduced amino acid sequence shows no homology to other known proteins; the C terminus, however, resembles those of other protein metallo-carboxypeptidase inhibitors, suggesting a common mechanism of inhibition. Recombinant TCI expressed in Escherichia coli is fully functional and inhibits carboxypeptidases of the A/B subfamily with equilibrium dissociation constants in the nanomolar range. Structural analyses by circular dichroism and nuclear magnetic resonance indicate that TCI is a protein strongly constrained by disulfide bonds, unusually stable over a wide pH range and highly resistant to denaturing conditions. As a tight binding inhibitor of plasma carboxypeptidase B, also known as thrombin-activatable fibrinolysis inhibitor, recombinant TCI stimulates fibrinolysis in vitro and thus may have potential for applications to prevent or treat thrombotic disorders.

The biological actions of many proteases are tightly controlled by specific interactions with proteinaceous inhibitors (1, 2). In contrast to the wide variety of structurally and functionally diverse inhibitors of endoproteases (3, 4), however, only few specific inhibitors that bind to metallo-carboxypeptidases have been identified. The inhibitors described so far are from Solanaceae (tomato and potato; 38–39 residues) (5, 6), the intestinal parasite Ascaris suum (65 residues) (7), the medical leech Hirudo medicinalis (66 residues) (8), and rat and human tissues (223 residues) (9, 10). These carboxypeptidase inhibitors differ in their mechanism of action; whereas the inhibitor from mammalian tissues apparently binds to carboxypeptidases through a small region that shows sequence similarity to the inhibitory loop present in their propeptidases (9, 11, 12), all of the others known rely on the interaction of their C-terminal tail with the active site groove in a way that mimics substrate binding (13).

In recent years the interest in carboxypeptidase inhibitors has been fostered by the identification of nondigestive carboxypeptidases in extrapancreatic tissues and fluids that possess diverse physiological functions (13, 14). In particular, two carboxypeptidases have been found in plasma, i.e. carboxypeptidase (CP)\(^1\) N (15) and plasma CPB, also known as thrombin-activatable fibrinolysis inhibitor (TAFI) (16, 17). CPN is a constitutively active enzyme that cleaves numerous endogenous vasoactive peptides and proteins such as kinins and anaphylatoxins (18). TAFI circulates in plasma as a zymogen; the activated form TAFIa down-regulates fibrinolysis presumably by removing C-terminal lysine residues from fibrin that is already partially digested by plasmin (19). Removal of these residues, which act as ligands for the lysine-binding sites of plasminogen and tissue-type plasminogen activator (tPA), results in a reduced plasminogen activation by tPA and consequently in a prolongation of clot lysis time (20). This mechanism provides a rationale for inhibiting TAFIa to enhance clot lysis. Indeed, experiments performed both in vitro and in vivo have shown that the potato carboxypeptidase inhibitor (PCI) significantly enhances clot lysis induced by tPA via the inhibition of TAFIa (21, 22). Similar effects have recently been demonstrated for the leech carboxypeptidase inhibitor (LCI) (2). Taken together, these data suggest a potential application of carboxypeptidase inhibitors as pro-fibrinolytic agents or lead compounds for the prevention or treatment of thrombotic disorders.

1 The abbreviations used are: CP, carboxypeptidase; LCI, leech carboxypeptidase inhibitor; MALDI-TOF MS, matrix-assisted laser/desorption ionization-time of flight mass spectrometry; PCI, potato carboxypeptidase inhibitor; rPCI, recombinant PCI; RACE, rapid amplification of cDNA ends; HPLC, high performance liquid chromatography; TAFI, thrombin-activatable fibrinolysis inhibitor; TCI, tick carboxypeptidase inhibitor; rTCI, recombinant TCI; TM, thrombomodulin; tPA, tissue-type plasminogen activator; MES, 4-morpholinethanesulfonic acid.

2 S. Salamanca, J. Lorenzo, A. Rovira, M. Borrell, J. Vendrell, F. X. Aviles, H. Fritz, and C. P. Sommerhoff, manuscript in preparation.

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Blood-sucking animals such as leeches, insects, and ticks are known to contain complex mixtures of compounds that interfere with their host’s hemostatic system, allowing fast and successful feeding of blood (23, 24). Several protein inhibitors of serine proteases have been isolated from leeches and ticks that include anticoagulants such as thrombin and factor Xa inhibitors (25, 26) as well as others whose biological functions are not yet fully understood (27, 28). In the present study, we have detected, isolated, cloned, expressed, and characterized a novel carboxypeptidase inhibitor from the ixodid tick Rhipicephalus bursa. This protein, named tick carboxypeptidase inhibitor (TCI), is the first carboxypeptidase inhibitor found in ticks. Most of TCI does not show sequence similarity with other known proteins; its C terminus, however, resembles those of the inhibitors from Solanacea, Aescaris, and Hirudo, suggesting a common mechanism of action. TCI inhibits different carboxypeptidases of the A/B subfamily with nanomolar affinities. Moreover, the generated recombinant form of the inhibitor (rTCI) accelerates fibrinolysis—10 times more effectively than rPCI and thus may have potential for applications in coagulation disorders.

EXPERIMENTAL PROCEDURES

Materials—R. bursa ticks were collected from cows and sheep in Catalonia, immediately frozen in liquid nitrogen, and stored at −80 °C. Restriction enzymes, ligases, and DNA polymerases were obtained from Roche Applied Science and Exogen. Roche Applied Science synthesized all of the oligonucleotides. Dithiothreitol, guanidine hydrochloride, reduced glutathione, and oxidized glutathione were purchased from Sigma. Recombinant human CPA1, CPA2, and CPB were a generous gift from Prof. J. J. Enghild (University of Copenhagen). Recombinant PCI was produced and purified as described previously (32), three oligonucleotides were designed using Escherichia coli codon usage: A, 5′-AAGARGTGYGTISWAARGDITGG-3′ (corresponding to nucleotides 3442–3450 of the complete cDNA; see Fig. 1) and gene-specific primer 2, 5′-TGGATCCACGAGCAGACATGGTGCAGCC-3′ (corresponding to nucleotides 420–429 of the complete cDNA). After second strand cDNA synthesis and ligation of the adaptor oligonucleotide, “semi-nested” PCR was performed with the adaptor primer mix and gene-specific primers 1 and 2 in a first and second round of PCR, respectively. The PCR products were analyzed and cloned as described above.

Construction of the TCI Expression Vectors—PCR was used to append the 5′ bases coding for the N-terminal amino acids of mature TCI to the TCI cDNA obtained by 3′-RACE. Two oligonucleotides were designed using N- and R1 5′-CCGAATTCATGAAAAAGACAG-3′ (corresponding to nucleotides 444–449 of the complete cDNA; see Fig. 1) and gene-specific primer 2, 5′-TGGATCCACGAGCAGACATGGTGCAGCC-3′ (corresponding to nucleotides 420–429 of the complete cDNA). After second strand cDNA synthesis and ligation of the adaptor oligonucleotide, “semi-nested” PCR was performed with the adaptor primer mix and gene-specific primers 1 and 2 in a first and second round of PCR, respectively. The PCR products were analyzed and cloned as described above.

For the cloning of this semisynthetic TCI cDNA into the expression vector pBAT-4 (32), three oligonucleotides were designed using E. coli codon usage: C, 5′-CCGAATTCATGAAAAAGACAG-3′; D, 5′-TGACCTGTTGCTTGTCGTGCAAGCAGACCGA-3′; and E, 5′-CCGAATTCATGAAAAAGACAG-3′. The PCR product obtained by successive two PCR cycles (conditions see above) using the oligonucleotides D and E, respectively, to introduce the BamHI signal sequence was cleaved with EcoRI and HindIII and ligated with the BamHI vector pBAT-4 to generate pBAT-4-TCI. The pBAT-4-OmpA-TCI vector was transformed into E. coli strain BL21(DE3). For the cloning of rPCI into expression vector pBAT-4-TCI was used to transform E. coli strains BL21(DE3) and B834.

Heterologous Expression of TCI—For the production of rTCI, an overnight culture of E. coli BL21(DE3)/pBAT-4-OmpA-TCI grown in Luria-Bertani medium was used to inoculate 0.5 liter of M9CAS medium containing 0.5% glycerol. At an OD600 of 1 the culture was induced with 1 mM isopropyl-β-D-thiogalactopyranoside. After 24 h of induction the culture was centrifuged, and the supernatant was applied onto a Sep-Pak C18 cartridge (Waters), and rPCI was recovered from the gel and cloned into the pGEM-T Easy vector (Promega) to generate the clone pGEM-TCI-N3/R1.

For the intracellular expression of rTCI, BL21(DE3)/pBAT-4-OmpA-TCI grown in Luria-Bertani medium and used to inoculate 0.5 liter of the same medium. At an OD600 of 0.8–1.0, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM. After 8 h of induction the culture was centrifuged, and cells were sonicated in Tris-HCl buffer (20 mM, pH 8.5) containing 0.5 mM EDTA. After centrifugation the pellet was washed with Tris-HCl buffer containing 0.5 mM EDTA and 2% Triton X-100, dissolved in Tris buffer containing 6 mM guanidine hydrochloride and 30 mM dithiothreitol, and stored at −80 °C.
kept for 6 h at 20 °C. Subsequently, the sample was dialyzed against Tris buffer containing 1 mM reduced and 0.5 mM oxidized glutathione for 24 h before measurements of CP inhibitory activity.

**Molecular Mass Determination, Carboxymethylation, and Amino Acid Sequencing**—The molecular masses of natural and of recombinant TCI were determined by MALDI-TOF MS on a Bruker Ultraflex spectrometer. Samples were prepared by mixing equal volumes of the protein solution and matrix solution (sinapinic acid in 30% acetonitrile with 0.1% trifluoroacetic acid). To determine the number of cysteines the inhibition of bovine CPA. The majority of the inhibitory activity was recovered in the fraction eluted at 20% acetonitrile. The material was subsequently loaded at intermediate pH onto an affinity column prepared by immobilization of bovine CPA on a Sepharose 4B resin. The inhibitor was strongly bound and could only be eluted using an extremely alkaline pH (phosphate buffer, pH 12); the recovery of the active inhibitor at such an extreme pH indicated a strong affinity toward CPA and a high stability of the protein. In a final reversed phase HPLC step a virtually homogeneous TCI preparation was obtained in a peak with a retention time of ~31 min (10–60% linear acetonitrile gradient over 60 min). Using this procedure, ~2 µg of TCI were isolated from 200 ticks.

The purified TCI was analyzed by automated Edman degradation using a Beckman LF3000 Protein Sequencer.

**Circular Dichroism and NMR Spectroscopy**—Samples for CD spectroscopy were prepared by dissolving the protein in 0.5 mg/ml in 0.1% aqueous trifluoroacetic acid (pH 2.0) or sodium guanidine hydrochloride and 30 mM dithiothreitol for 2 h at 50 °C. Carboxymethylation of cysteines was achieved by the addition of 0.5 M iodoacetamide for 1 h at room temperature in the dark. The solution was diluted 10 times with water and analyzed using the same spectrometer. N-terminal amino acid sequence analysis was carried out by automatic Edman degradation using a Beckman L-8000 Protein Sequencer.

**RESULTS**

**Purification, Protein Sequence Analysis, and Mass Determination of TCI**—TCI was isolated from R. bursa ticks using a combination of reverse phase and affinity chromatography. A crude homogenate was prepared from 200 adult specimens, clarified by centrifugation, and injected into a reverse phase cartridge (Sep-Pak C18). The fractions were eluted with increasing acetonitrile concentrations, evaporated, and tested for inhibition of bovine CPA. The majority of the inhibitory activity was recovered in the fraction eluted at 20% acetonitrile. The material was subsequently loaded at intermediate pH onto an affinity column prepared by immobilization of bovine CPA on a Sepharose 4B resin. The inhibitor was strongly bound and could only be eluted using an extremely alkaline pH (phosphate buffer, pH 12); the recovery of the active inhibitor at such an extreme pH indicated a strong affinity toward CPA and a high stability of the protein. In a final reversed phase HPLC step a virtually homogeneous TCI preparation was obtained in a peak with a retention time of ~31 min (10–60% linear acetonitrile gradient over 60 min). Using this procedure, ~2 µg of TCI were isolated from 200 ticks.

The purified TCI was analyzed by automated Edman degradation and MALDI-TOF MS. The sequence NHE-NECVSKGF-GCLPQSDCPCQEARLSYGGCSTV was generated, purified, and cloned. Sequence analysis of the plasmid showed the presence of a 3′-cDNA fragment (nucleotides 166–494 of the complete cDNA; Fig. 1). This partial cDNA sequence was used to design two specific oligonucleotides for 5′-RACE. A fragment of ~450 base pairs was amplified by
nested PCR and cloned. Seven clones were sequenced that all contain fragments representing nucleotides 1–420 of the TCI cDNA. As shown in Fig. 1 the full-length TCI cDNA consists of 494 nucleotides and contains a 66-nt 5′-untranslated sequence, an open reading frame of 291 nucleotides, and a 137-nt 3′-untranslated region. A canonical polyadenylation signal AATAAA is found 16 nucleotides upstream the poly(A) tail. The deduced protein consists of 97 amino acid residues. A 22-residue hydrophobic signal peptide, predicted by the program SignalP (34), precedes the N-terminal Asn residue found in the TCI protein isolated from ticks. With 7935 Da the calculated molecular mass of the deduced mature protein (75 amino acid residues) is larger than that of natural TCI determined by MS (7798 Da). The observed difference of 137 Da corresponds to the mass of the C-terminal His75 residue that most likely is removed by proteolytic processing during the CPA affinity chromatography. Computer search analyses did not show significant homology with any deposited amino acid or nucleotide sequence.

Heterologous Expression of rTCI—PCR was used to generate the 5′-bases that are missing in the partial cDNA obtained by 3′-RACE and code for the 20 N-terminal amino acid residues of mature TCI. The resulting semi-synthetic cDNA was ligated into the expression vector pBAT-4 (see “Experimental Procedures” for details) that was previously utilized for the recombinant production of PCI and LCI (29). The resulting expression plasmid pBAT-4-OmpA-TCI was used to transform E. coli strain BL21(DE3). After induction of expression in shaker flasks, recombinant TCI was predominantly detected in the culture medium, suggesting that the OmpA signal peptide correctly directed the immature protein through the plasma membrane into the bacterial periplasmic space with concomitant removal of the signal peptide and subsequent release of mature rTCI through the outer membrane into the culture medium. Approximately 2 mg of rTCI/liter of supernatant were produced, of which 40–50% was recovered by purification using reverse phase and cation exchange chromatography (see “Experimental Procedures”). In this recombinant preparation several rTCI species were detected by reversed phase HPLC; these species most likely reflect different folding intermediates because one predominant species was obtained after treatment with reduced/oxidized glutathione (pH 8.5, 24 h), which showed a chromatographic retention time similar to that of natural TCI isolated from ticks. N-terminal amino acid sequence analysis and MALDI-TOF MS (7935 Da) verified the identity and correct processing of rTCI. In addition, MS analysis after treatment with the alkylating reagent vinylpyridine showed no incorporation of vinylpyridine groups, suggesting that all of the cysteine residues of this rTCI preparation are involved in disulfide bond formation.

During optimization of rTCI production intracellular expression systems were also examined. Unfortunately, neither soluble intracellular TCI nor inclusion bodies were detectable after recombinant expression using the systems BL21(DE3)/pBAT-4-TCI or B834/pBAT-4-TCI.

Conformational Properties of rTCI—The structural features of recombinant TCI were analyzed using CD and NMR spectroscopy. rTCI displays a peculiar CD spectrum with a well defined minimum of ellipticity at 204 nm (Fig. 2A). This band may be related to the presence of a high percentage of residues in β-structures and loops (35). CD measurements at different pH values show that rTCI essentially has the same structure at pH 2.0, 6.5, and 8.0; the obtained spectra are virtually identical. The CD spectrum of TCI is similar to those of PCI and LCI (8, 36) but does not possess a maximum of ellipticity at 228 nm; the presence of this 228-nm band was attributed to a charac-
teristic environment of the Tyr residue located at the C terminus of both PCI and LCI.

CD spectroscopy measurements have shown that the degree of denaturation of PCI and LCI correlates with the disappearance of their characteristic CD ellipticity bands (8, 36). The 204-nm band of TCI maintains most of its intensity when the temperature is raised from 25 to 90 °C, and the native spectrum is completely recovered after lowering the temperature back to 25 °C. Even treatment with 6 M guanidine hydrochloride does not significantly affect the characteristic spectrum of TCI (Fig. 2B). However, the reducing agent dithiothreitol (10 mM) strongly decreases the intensity of the 204-nm band, and this effect is even stronger in the presence of denaturants, causing almost complete disappearance of such a band.

1H NMR spectra of rTCI showed a wide signal dispersion of resonances at both the low (amide and aromatic region) and high fields (methyl region) with a significant number of potential methyl protons in the 0–1-ppm region and rich in exchangeable resonances in the NH region (Fig. 3). A high resistance of NH protons to the exchange with the solvent was detected after raising the temperature from 25 to 90 °C in H2O. In contrast, most of these protons were already exchanged at 25 °C in D2O (Fig. 3). Interestingly, at high temperatures the shape/disposition of the spectra in the 0–1-ppm region was very similar to that at 25 °C in both H2O and D2O. The two-dimensional NMR analysis of rTCI also showed a significant resistance of nuclear Overhauser effect contacts against temperature. Thus, as shown in Fig. 4, the fingerprint region is similar at 25 and 75 °C in 10% D2O. Taken together, CD and NMR analyses indicate a high conformational thermostability and resistance against denaturants of rTCI, which requires the presence of reducing agent to unfold.

**Inhibitory Activity and Selectivity of TCI—Equilibrium dissociation constants for the complexes of rTCI with different metallo-carboxypeptidases were determined using a pre-steady-state approach.** It is deduced that rTCI is a tight binding, competitive inhibitor of different carboxypeptidases of the A/B subfamily with $K_i$ values in the nanomolar range (Table I). Compared with rPCI, the affinity of rTCI toward porcine CPB and human TAFIa is ~5-fold higher, whereas bovine CPA and human CPB are inhibited with similar $K_i$ values. rTCI is also ~2-fold better inhibitor toward CPA2 and bovine TAFIa than the potato inhibitor. Both rTCI and rPCI have no effect on the enzymatic activity of the plasma "regulatory carboxypeptidase" CPN even at a much higher concentration than in the assays with the other carboxypeptidases (100 μM versus ~10 nM). Titration experiments suggest that both rTCI and rPCI inhibit bovine CPA and TAFIa with a 1:1 stoichiometry and that the recombinant proteins are fully functional (specific activity >90% of the theoretical values).

It is worth mentioning that the $K_i$ values determined using rTCI and TCI isolated from ticks are virtually identical (data not shown), verifying that the recombinant protein is correctly folded. In addition, these results demonstrate that the inhibitory activity of TCI is not significantly affected by the presence of the C-terminal His7 residue that, supposedly, is removed by proteolytic processing during the isolation of TCI from ticks.

**Effect of rTCI on the tPA-induced Lysis of Clots—Clot formation and lysis assays were performed to compare the effectiveness of rTCI and rPCI to inhibit TAFIa during fibrinolysis. Clots lysis was induced by the addition of 1.2 nM tPA in the absence and presence of either rPCI (Fig. 5A) or rTCI (Fig. 5B).** In this assay TM enhances the activation of TAFI by thrombin (38), thus prolonging clot lysis time (Fig. 5, curves with open symbols). High concentrations of either rPCI or rTCI restore clot lysis time to that observed in the absence of TM (see closed triangles) because their pro-fibrinolytic effect is due to the inhibition of TAFIa. However, depending on the concentration used, PCI either prolongs (circles) or shortens (triangles) lysis, which has been explained by its effect on both the activity and the stability of TAFIa (39). rTCI has a similar biphasic effect,

![Image 125x545 to 497x737]

**Fig. 4. Fingerprint region of the nuclear Overhauser effect spectra of rTCI.** Two-dimensional NMR spectra were obtained both at 25 and 75 °C at a protein concentration of 1 mM in 10% D2O. The spectra are unsymmetrized.

### Table I

| Carboxypeptidase      | $K_i$ (nM) |
|-----------------------|-----------|
| **rTCI**              | **rPCI**  |
| Bovine CPA            | 1.1 ± 0.3 | 1.6 ± 0.2 |
| Human CPA1            | 1.2 ± 0.4 | 1.6 ± 0.3 |
| Human CPA2            | 3.6 ± 0.5 | 8.8 ± 0.7 |
| Porcine CPB           | 1.6 ± 0.3 | 7.2 ± 0.6 |
| Human CPB             | 1.3 ± 0.2 | 1.8 ± 0.3 |
| Bovine TAFIa          | 1.3 ± 0.3 | 2.5 ± 0.4 |
| Human TAFIa           | 1.2 ± 0.4 | 5.3 ± 0.5 |
| Human CPN             | ≈         | ≈         |

*No inhibition at 100 μM concentration of inhibitor.*

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but the prolongation of fibrinolysis is much less pronounced than that caused by rPCI.

To compare the effects of rTCI and rPCI on fibrinolysis, clot lysis times were calculated as the time required for 50% clot lysis. Fig. 6 shows the effects of rPCI (panel A) and rTCI (panel B) on clot lysis induced by either 1.2 or 2.4 nM tPA. Depending on the concentration used, both rTCI and rPCI prolong or shorten lysis time. The anti-fibrinolytic potential of rPCI is substantial; at a concentration of 20 nM, rPCI accelerates clot lysis at higher concentrations (1000 nM, ▼). B, compared with rPCI, low concentrations of rTCI (2 nM, ●) retard fibrinolysis to a much smaller extent whereas higher concentrations (100 nM, ▼) accelerates lysis comparable with rPCI.

DISCUSSION

In the present study we have isolated, cloned, expressed, and characterized a novel metallo-carboxypeptidase inhibitor, named TCI, from the ixodid tick R. bursa. TCI is a 75-residue protein rich in glycines (10 residues) and cysteines (12 residues), the latter involved in the formation of six disulfide bridges. The cDNA of TCI was obtained by a combination of 3'- and 5'-RACE approaches; it contains an open reading frame coding for a protein of 97 residues that consists of a hydrophobic signal sequence (22 residues) preceding mature TCI. A signal peptide cleavage site is predicted between Ala1 and Asn1 (Fig. 1), verifying the N-terminal asparagine residue obtained by the N-terminal amino acid sequencing of TCI isolated from ticks. The mature protein deduced from the cDNA contains an additional C-terminal His75 residue that was not detected by MALDI-TOF MS of the isolated protein and most likely is proteolytically removed during affinity purification from the tick extract (see below). The cDNA and amino acid sequences of TCI do not show significant similarity to those of known proteins.

For recombinant periplasmatic expression in E. coli, a semi-synthetic gene coding for a fusion protein of the OmpA signal sequence and mature TCI, was constructed by PCR based on the 3'-RACE cDNA clone. The expression yield was lower than those observed for rPCI and rLCI using the same expression system: 2 mg versus 5 and 11 mg/liter of culture, respectively.
(29), most likely reflecting the higher number of disulfide bridges of TCI (six versus three and four in PCI and LCI, respectively). Indeed, during purification several rTCI species were detected by reversed phase HPLC that appear to be folding intermediates because only one predominating species accumulated after treatment with reduced/oxidized glutathione. The elution profile and the inhibitory properties of this recombinant protein are very similar to those of the naturally occurring inhibitor, confirming that rTCI is correctly folded and functionally equivalent to the protein isolated from ticks. In addition, titration experiments indicate that rTCI displays a fully inhibitory capability. Attempts to improve rTCI yields, e.g., by using an intracellular E. coli expression system, failed so far; neither soluble rTCI nor inclusion bodies were detectable in the cytoplasm.

CD and NMR studies demonstrate that rTCI retains a well folded conformation over a wide range of pH and temperatures and is unusually resistant to denaturing conditions. The CD spectra of this small disulfide-rich molecule suggest a high content in residues forming β-structures and/or loops, in agreement with its richness in glycine and cysteine residues. However, the anomalous dichroic bands that are similar to those reported for PCI and LCI hinder conformational predictions (8, 36). TCI seems to possess few secondary structures that could be involved in the formation of an internal core, because the protons in the NH NMR region exchange with the solvent (D,O) on a very short time scale at low temperatures. Thus, the observed maintenance of a proper conformation under extreme pH and temperature conditions appears to be dependent primarily on the establishment of disulfide bonds that constrain its structure.

TCI is a tight binding inhibitor that inhibits different metallo-carboxypeptidases with equilibrium dissociation constants in the nanomolar range. Several reasons provide support for the hypothesis that the C terminus of TCI is the primary binding site for binding to these carboxypeptidases. First, the C-terminal residue of TCI as well as those of PCI and LCI (His, Gly, and Glu, respectively) is removed after binding to carboxypeptidase. Despite the absence of such C-terminal residue, the activity of these inhibitors is unaltered. Secondly, the C-tail of TCI is similar to those of other carboxypeptidase inhibitors in amino acid composition and certain sequence features (Fig. 7). This similarity observed in otherwise completely different molecules that are derived from evolutionary highly distant species, i.e., blood-sucking animals and Solanacea plants, is likely to result from convergent evolution dictated by the three-dimensional structures of the target metallo-carboxypeptidases. X-ray crystallographic studies have shown that only two residues of PCI and LCI are located within the active site of the inhibited carboxypeptidase, i.e., the penultimate Tyr and the newly generated C-terminal Val residue, with the latter playing a key role in the inhibition because its carboxylate group is coordinated to the catalytic zinc of the enzyme (37, 41). Similarly, mutagenesis studies using PCI have indicated that Tyr and Val are important residues in the interaction with the carboxypeptidase, whereas the preceding Pro mainly contributes to maintain the rigidity of the tail (42, 43). Tyr and Val can be substituted by another aromatic residue and Leu, respectively, without loss of inhibitory capability. Thus, after removal of the His10 residue, the C-terminal Trp3 and Leu30 of des-His75-TCI could mimic the Tyr and Val present in PCI and LCI, resulting in a similar conformational arrangement in the complex with a carboxypeptidase (Fig. 8).

Finally, TCI binds and inhibits carboxypeptidases of the A/B subfamily without affecting CPN, a carboxypeptidase that belongs to the "regulatory" subfamily (also known as subfamily NH). This is consistent with the behavior of other protein carboxypeptidase inhibitors (44). The crystal structure of the duck CPD domain II, another regulatory carboxypeptidase subfamily member, reveals a long loop that shapes the border of the funnel into the active site and probably binds any of these inhibitors through their C-termini (45).

It has been well established that the inhibition of TAFIa by PCI affects tPA-induced clot lysis both in vitro and in vivo (21, 22, 46). More recently, PCI and the synthetic inhibitor 2-guanidinobenzamidincaptoacetic acid have been shown to either accelerate or inhibit fibrinolysis depending on the concentration used, which has been explained by a dual effect on the activity and the stability of TAFIa (39, 40). Motivated by the higher affinity of rTCI compared with (r)PCI toward TAFIa, we have also compared their fibrinolytic activity. The results (Figs. 5 and 6) show that both inhibitors have a biphasic effect on tPA-mediated clot lysis of human plasma. However, rTCI is more potent that rPCI, i.e., both ~10-fold lower concentrations are required to accelerate fibrinolysis, and the pro-fibrinolytic effect at high concentrations is more pronounced. These differences may in part originate from the ~5-fold higher affinity of TCI compared with PCI toward TAFIa (Table 1). More importantly, compared with PCI, rTCI has only a minor pro-

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3 J. L. Arolas, S. Bronsoms, S. Ventura, and F. X. Aviles, unpublished data.
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fibrolytic effect at low concentrations. This may result from a higher stability of the rTCI-TAFIa complex and reflect the higher stability of the rTCI/TAFI complex and its therapeutic potential as an adjuvant to more efficient secondary contacts with the enzyme. Based on these results rTCI has therapeutic potential as an adjuvant to thrombotic disorders (47–49).

Considering the few carboxypeptidase inhibitors identified so far, it is interesting to note that TCI together with LCI, an inhibitor previously isolated from the medical leech (8), is the second inhibitor of metallo-carboxypeptidases identified in hematophagous ectoparasites. These species are well known to contain a variety of serine proteinase inhibitors (25, 26), most prominently among them the thrombin and factor Xa inhibitors that are potent anticoagulants. It appears likely that TCI and LCI contribute to the maintenance of blood liquidity during feeding and inside the animal by stimulation of fibrinolysis via secretion of plasminogen activators in their saliva (50). In addition, TCI and LCI may modulate inflammation and host defense mechanisms, e.g. by hindering the inactivation of kinins by TAFIa (51–53), thus increasing blood flow at the site of the bite, or by affecting the activity of CPA released from mast cell that are activated during parasitic infections (54).

Acknowledgments—We thank M. Rodríguez and S. Streicher for technical assistance and helpful comments and S. Prieto for help in the collection of ticks. We gratefully acknowledge the generous gift of bovine and human TAFI from J. Enghild and Z. Valnickova.

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34. TAFI contribute to the maintenance of blood liquidity during feeding and inside the animal by stimulation of fibrinolysis via inhibition of TAFIa. Similarly, vampire bats rely on the stimulation of fibrinolysis rather than the inhibition of coagulation during feeding by secretion of plasminogen activators in their saliva (50). In addition, TCI and LCI may modulate inflammation and host defense mechanisms, e.g. by hindering the inactivation of kinins by TAFIa (51–53), thus increasing blood flow at the site of the bite, or by affecting the activity of CPA released from mast cell that are activated during parasitic infections (54). Such mechanisms to modulate inflammation and host defense may be particularly relevant for leeches and hard ticks (Family Ixodidae) that feed for several days or even weeks with its mouthpart embedded in their vertebrate hosts.
A Carboxypeptidase Inhibitor from the Tick *Rhipicephalus bursa*: ISOLATION, cDNA CLONING, RECOMBINANT EXPRESSION, AND CHARACTERIZATION

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*J. Biol. Chem. 2005, 280:3441-3448.*
doi: 10.1074/jbc.M411086200 originally published online November 23, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M411086200

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