DOI: 10.1002/anie.201103262

Peptide-Bond Synthesis

Structural Evidence for Standard-Mechanism Inhibition in Metallopeptidases from a Complex Poised to Resynthesize a Peptide Bond**

*Joan L. Arolas, Tiago O. Botelho, Andreas Vilcinskas, and F. Xavier Gomis-Rüth**

Dedicated to Professors Robert Huber and Wolfram Bode on the occasion of their 75th and 70th birthday, respectively

Metallopeptidases (MPs) are part of the virulence-factor armory secreted by pathogenic microorganisms, and among them are members of the thermolysin family of endopeptidases (family M4, MEROPS database).[1] Its prototype is thermolysin from *Bacillus thermoproteolyticus* (BtTL), which has important biotechnological applications in catalyzing the synthesis of peptide bonds in the presence of organic solvents.[2,3] Thermolysins are produced by entomopathogenic fungi such as *Metarhizium anisopliae*, which infects the greater wax moth (*Galleria mellonella*).[4] In response to infection, the moth produces the insect metalloproteinase inhibitor (IMPI).[5–7] Authentic IMPI purified from natural sources spanned 69 residues, was linked through disulfide bonds, and potently inhibited several thermolysins in addition to BtTL, but not trypsin, papain, or matrix metalloproteinases.[5–7] Besides IMPI, only *Streptomyces* metalloproteinase inhibitor (SMPI)[8] has been reported to be an efficient protein inhibitor of BtTL. However, no experimental information is available on the structural determinants of BtTL inhibition by any protein.

Mature IMPI (I20-S88, numbering as in Un*i*Prot P82176, plus three extra N-terminal residues, GMS) produced in *Escherichia coli* showed an inhibitory profile comparable to that of authentic IMPI and the form produced in insect cells (see Table S1 in the Supporting Information).[6,7] In addition, the inhibitor did not target a collection of MPs and peptidases of other classes (see Table S1 in the Supporting Information), so we conclude that IMPI is a specific nanomolar inhibitor of thermolysins. Mass-spectrometric analysis of IMPI after incubation with an equimolar amount of BtTL revealed a gain of 18 Da when compared with the freshly purified form (m/z: 7933.1 versus 7951.6) as a result of selective cleavage of a single peptide bond. In addition, incubation of IMPI with excess BtTL led to removal of the first three residues resulting from the cloning strategy, thereby giving rise to a single species of $m/z = 7675.6$ (I20-N56 + I57-S88; Figure 1a, upper spectrum). Accordingly, we found that IMPI is cleaved by BtTL in the same way as a substrate, but maintains its overall conformation because of the disulfide bonds. Furthermore, it also maintains its ability to form a complex with, and its inhibitory capacity against, BtTL (see [Eq. (1)]).

$$\rightarrow \text{peptide bond hydrolysis} \rightarrow$$

$$E + I \leftrightarrows E\text{-}I \leftrightarrows E\text{-}I^* \leftrightarrows E + I^* \qquad (1)$$

$$\leftarrow \text{peptide bond synthesis} \leftarrow$$

(E:enzyme; I and I*: intact and cleaved inhibitor)

The crystal structure of IMPI complexed with BtTL (see Table S2 in the Supporting Information for the crystallographic data) shows IMPI to display the overall shape of a spearhead of a rhombic base (Figure 1b, top left) and little regular secondary structure (Figure 1b). Structural coherence is achieved by five disulfide bonds and electrostatic interactions. The functionally most relevant part of IMPI is the six-residue "reactive-site loop" (P53–R58), whose outermost end at I57 features the tip of the spearhead. This loop is cleaved at N56–I57 in the crystal structure, as confirmed by mass-spectrometric analysis and the initial omit electron density map. The loop protrudes from the molecular structure and is linked through two flanking disulfide bonds to the subjacent "scaffold loop". The cleaved inhibitor is laterally inserted like a wedge into the active-site cleft of the peptidase (Figure 1c,d and see Figure S1a in the Supporting Information), which conforms to the general features previously described for closed BtTL structures.[9–11] Despite a nanomolar inhibition ($IC_{50} = 0.6$ nM), IMPI interacts with BtTL through a small surface, which is mainly derived from the reactive-site loop (see Table S3 in the Supporting Information). The latter runs across the active-site cleft in a substrate-like manner and the

[*] Dr. J. L. Arolas, Dr. T. O. Botelho, Prof. Dr. F. X. Gomis-Rüth
Proteolysis Lab; Molecular Biology Institute of Barcelona
CSIC, Barcelona Science Park
c/Baldiri Reixac, 15-21; 08028 Barcelona (Spain)
E-mail: fxgr@ibmb.csic.es
Homepage: http://www.ibmb.csic.es/home/xgomis
Prof. Dr. A. Vilcinskas
Institut für Phytopathologie und Angewandte Zoologie
Justus-Liebig-Universität Giessen
Heinrich-Buff-Ring, 26-32; 35392 Giessen (Germany)

[**] See Supporting Information for Acknowledgements, Abbreviations, and additional Concluding Remarks.

Supporting information for this article (details on the recombinant overexpression and purification of IMPI; inhibitory experiments of IMPI with a collection of peptidases of different classes; complex formation of IMPI with BtTL; assessment of the peptidyl-synthase activity of BtTL on IMPI; and crystallization and structure solution of the complex) is available on the WWW under http://dx.doi.org/10.1002/anie.201103262.

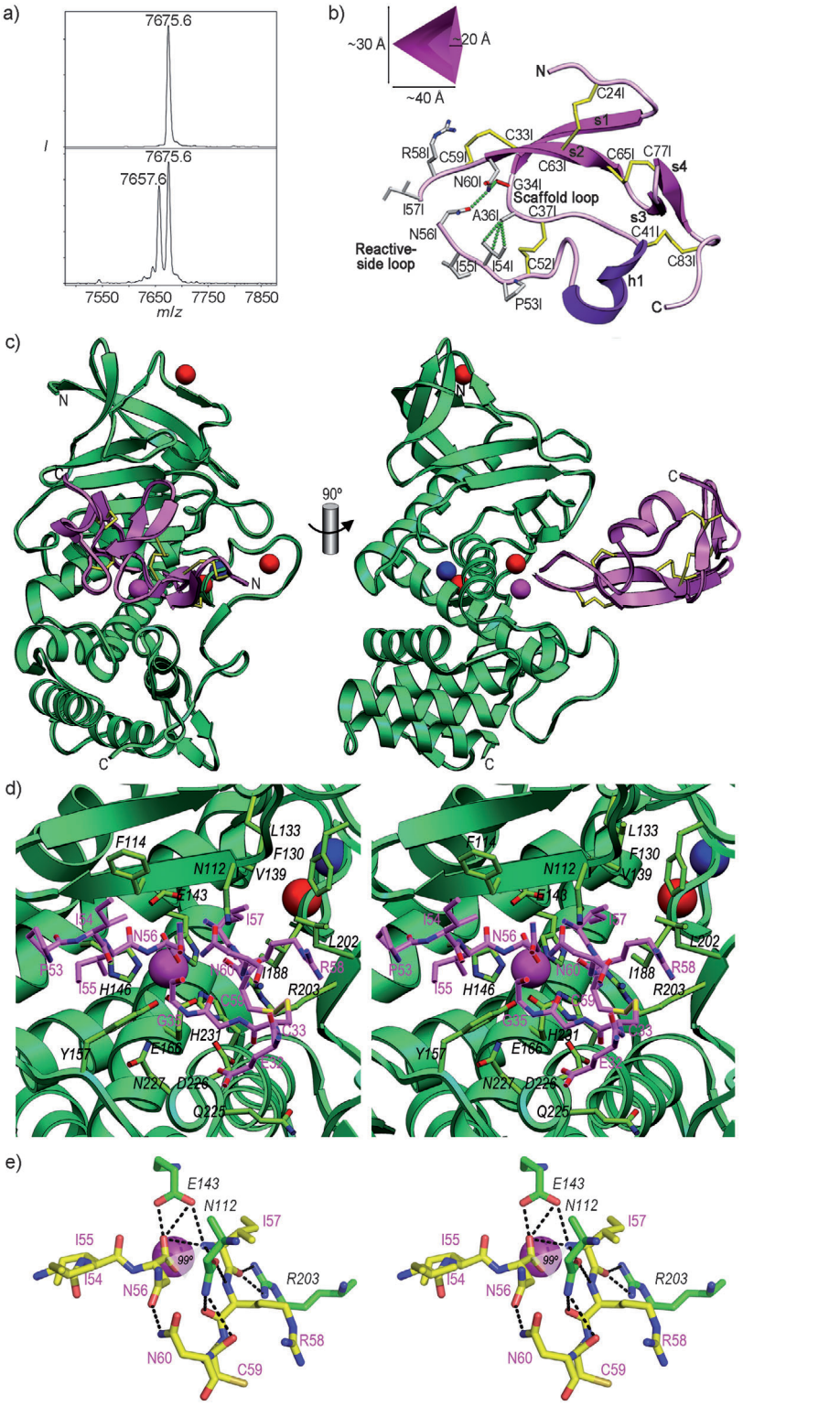

Figure 1. Structure of IMPI and of its BtTL complex. A) Mass spectrum of cleaved IMPI after incubation with excess BtTL (top); and in the presence of catalytic amounts of BtTL (bottom), which reveals partial resynthesis of a peptide bond (new signal at $m/z = 7657.6$). B) Ribbon plot of IMPI depicting the β strands (s1–s4) and a helical segment (h1). C) Front and lateral view of the complex of IMPI ((lilac) and BtTL (green). D) Close-up stereoview of (C) depicting only the reactive-site loop (P53–N60) and part of the scaffold loop (E32–G35) of IMPI (magenta labels) as a stick model with lilac carbon atoms. BtTL residues engaged in interactions with IMPI through side-chain atoms (see Table S3 in the Supporting Information) are shown with green carbon atoms. E) Stereoview of (D) depicting the termini of IMPI (yellow carbon model) to be rejoined, and the Bürgi–Dunitz trajectory of the attacking α-amino group of I57 on the target carbonyl group of N56.

chain termini resulting from bond cleavage are close to the catalytic zinc. Complex interactions of IMPI include antiparallel β-ribbon-like contacts with a β strand creating the upper rim of the active-site cleft of the enzyme (Figure 1 c,d). This strand is also contacted by the side chain of N56. In addition, the P_4 residue P53 is responsible for a sharp outwards kink of the preceding polypeptide chain; the P_3 residue I54 establishes a hydrophobic interaction with the ring of BtTL residue *F114* (enzyme residues in italics); I55 is accommodated in the S_2 pocket shaped by *H146*, *Y157*, and *W115*; and the C-terminal carboxylate group of N56 interacts with the catalytic zinc ion in an asymmetric bidentate manner (Figure 1 d,e and Table S3 in the Supporting Information). This carboxylate further contacts the side chain, *E143*, of the general base/acid for catalysis,[12] thus necessitating that one of the acidic groups must be protonated. On the primed side of the cleft, I57 nestles into the hydrophobic S_1' pocket. Its α-amino group forms a hydrogen bond with the carbonyl group of upper-rim strand *A113*, and its carbonyl oxygen atom interacts with *R203*. *N112* approaches both the amide nitrogen atom and the carbonyl oxygen atom of R58 in P_2', as well as the side chain of N60. As in other structures of BtTL with occupied primed sides of the cleft (e.g. PDB 4TMN and 3TMN), the peptide chain is bent approximately 90° out of the cleft at I57 as a consequence of the aforementioned interactions of IMPI with *R203* and *N112* of BtTL (Figure 1 d). Notably, the ligand chains of a P_1'-P_2' single-product complex (PDB 3TMN) and the Z-Phe moiety of compound Z-Phe^P-Leu-Ala, which mimics a reaction intermediate occupying subsites S_2–S_2' (PDB 4TMN), adopt similar positions to those of I57–R58 and I55–N56 of IMPI, respectively. Thus, the present structure represents the first two-product complex of BtTL—understood as a complex containing the upstream and downstream cleavage products—covering the cleft from S_4 to S_2', as may occur in

genuine peptidic substrates after cleavage (intermediate E–I* in [Eq. (1)]. In addition, this is an exceptional case for MP inhibitors: while several serine peptidase (SP) protein inhibitors (SPIs) are modified by their cognate enzymes, metalloendopeptidase protein inhibitors are generally not cleaved upon formation of a complex.

An overlay of the structure of BtTL/IMPI on the structures of two BtTL complexes with reaction-intermediate analogues further revealed that the carboxylate group of N56 coincides with the PO_2 group of the analogues and that the Cα atom of I57 is practically superimposable on the Cα atom of the leucine residues of the phosphorus compounds (see Figure S1 b in the Supporting Information). In addition, the N terminus of I57 is very close to the carboxylate carbon atom of N56 (Figure 1 d,e and Figure S1 b in the Supporting Information) and a simple rotation of about 40° around the C–Cα bond of I57 would allow formation of an amide bond between these residues. Indeed, when analyzing the Bürgi–Dunitz trajectory of the potential nucleophilic attack of the α-amino group of I57 on the target carbonyl group of N56—that is, the one that is not coordinated by the general base of BtTL, namely *E143*—a value of 99° is obtained, which is in the range described for such reactions (105° ± 5°; see Figure 1 e and Ref. [13]). Accordingly, the present structure also represents an enzyme–substrate complex poised for synthesis of a peptide bond. To test this hypothesis purified cleaved IMPI (Figure 1 a; top) was incubated with catalytic amounts of BtTL for 24 h, which resulted in about 35 % of the sample losing 18 Da in mass (Figure 1 a; bottom), which indicates that the peptide bond at position N56–I57 was rejoined. We thus showed that the hydrolytic reaction of protein substrates by BtTL is reversible, according to [Eq. (1)], that is, BtTL has peptidyl synthase activity on folded protein substrates. Peptidyl synthase activity had been previously shown for BtTL, generally for small peptides and in the presence of high concentrations of organic solvent only (which shifts the pK_a' value of the N-terminal substrate carboxylate group to promote water abstraction[2,3]) and specifically for cleaved SMPI.[14] Finally, no significant differences in the structures were observed between BtTL when complexed with IMPI and the reaction-intermediate analogues or cleavage product for the active site.[12] This finding indicates that, once the Michaelis complex has been formed after closure of the cleft, the forward hydrolytic and reverse synthetic reactions [Eq. (1)] can occur with minimal motion, that is, with little energetic penalty. This offers an explanation for the reversibility of the reaction.

IMPI is similar in structure to proteins from invertebrate members of MEROPS inhibitor family I8.[15] Such proteins are SPIs, the only exception being IMPI, and this is reflected by differences in the length and sequence of their reactive-site loops (see Table S4 in the Supporting Information). The closest in structure is the chymotrypsin/cathepsin G inhibitor from the honeybee, *Apis mellifera* (PDB 1CCV; Figure S1 c in the Supporting Information). Among these inhibitors, Michaelis complexes with target SPs are only available for *Ascaris suum* chymotrypsin/elastase inhibitor (PDB 1EAI) and *Ancylostoma caninum* nematode anticoagulant protein 5 (PDB 2P3F). These complexes show an intact inhibitor bound to the enzyme. Overall, superposition of the structure of IMPI on its relatives resulted in only moderate sequence identity and structure similarity. Consistently, while the target complexes of SPIs of the I8 family reveal that these inhibitors bind similarly to IMPI, critical differences are found in the reactive-site loops. These findings strongly suggest IMPI evolved from SPIs of the I8 family by evolutionary adaptation of the reactive-site loop to specifically inhibit another class of peptidases.

Inhibitors of the I8 family follow the "standard mechanism" of inhibition, which has been demonstrated or suggested for over 19 inhibitor families of SPIs and for SMPI only as to MP inhibitors.[14–17] This mechanism foresees that a superficial reactive-site loop binds tightly with good fit in an extended, "canonical" conformation to the SP active-site cleft, which mimics a peptidic substrate.[17] This loop contains a scissile peptide bond, which is cleaved by the enzyme, but only very slowly.[16] The reaction is reversible and the complex can, thus, dissociate to yield either the cleaved or the intact form of the inhibitor. SPI families following this mechanism include Kazal, Kunitz, Bowman–Birk, and squash inhibitors,[15] which except for their reactive-site loops display different tertiary structures.[16] The inhibition mode of IMPI on BtTL conforms to these structural and chemical requirements, so the present complex provides the first structural evidence for standard-mechanism inhibition in MPs. In addition, this complex provides, to the best of our knowledge, the first example of a cleaved standard-mechanism inhibitor with a functional peptidase. This standard mechanism has some specific features for MPs: 1) specificity is not conferred by an S_1 pocket but by an S_1' pocket; 2) as in SPIs, the P_3-P_1 positions of the reactive-site loop of IMPI establish an antiparallel β-sheet interaction with the enzyme, but they contact the upper rim of the cleft instead of a floor-paving segment; 3) the fact that only cleaved IMPI was found after incubation with excess BtTL suggests that, although forward and reverse reactions occur in the active-site cleft of BtTL, the predominant species after dissociation is the cleaved one. This is in stark contrast with standard-mechanism SPIs: the half-life of intact bovine pancreatic trypsin inhibitor in the presence of trypsin spans several years.[18] These features point to significant differences in the cleavage kinetics of standard-mechanism inhibitors by MPs and SPs, which may be attributable to the fact that catalysis proceeds in two steps—which include a covalent acyl–enzyme intermediate—in the SPs, while MPs cleave substrates in a single step.[12]

Received: May 12, 2011
Published online: September 13, 2011

Keywords: enzymes · metallopeptidases · protein inhibitors · structure elucidation · thermolysin

[1] N. D. Rawlings, A. J. Barrett, A. Bateman, *Nucleic Acids Res.* **2010**, *38*, D227.
[2] M. Reslow, P. Adlercreutz, B. Mattiasson, *Eur. J. Biochem.* **1988**, *177*, 313.
[3] S. I. Wayne, J. S. Fruton, *Proc. Natl. Acad. Sci. USA* **1983**, *80*, 3241.

[4] P. Götz, V. Matha, A. Vilcinskas, *J. Insect Physiol.* **1997**, *43*, 1149.
[5] M. Wedde, C. Weise, R. Nuck, B. Altincicek, A. Vilcinskas, *Biol. Chem.* **2007**, *388*, 119.
[6] A. Clermont, M. Wedde, V. Seitz, L. Podsiadlowski, D. Lenze, M. Hummel, A. Vilcinskas, *Biochem. J.* **2004**, *382*, 315.
[7] M. Wedde, C. Weise, P. Kopacek, P. Franke, A. Vilcinskas, *Eur. J. Biochem.* **1998**, *255*, 535.
[8] S. S. Seeram, K. Hiraga, A. Saji, M. Tashiro, K. Oda, *J. Biochem.* **1997**, *121*, 1088.
[9] B. van den Burg, V. Eijsink in *Handbook of Proteolytic Enzymes, Vol. 1, 2nd ed.* (Eds.: A. J. Barrett, N. D. Rawlings, J. F. Woessner Jr.), Elsevier, London, **2004**, pp. 374.
[10] H. M. Holden, B. W. Matthews, *J. Biol. Chem.* **1988**, *263*, 3256.
[11] B. W. Matthews, J. N. Jansonius, P. M. Colman, B. P. Schoenborn, D. Dupourque, *Nature* **1972**, *238*, 37.
[12] B. W. Matthews, *Acc. Chem. Res.* **1988**, *21*, 333.
[13] H. B. Bürgi, J. D. Dunitz, E. Shefter, *J. Am. Chem. Soc.* **1973**, *95*, 5065.
[14] S. S. Seeram, K. Hiraga, K. Oda, *J. Biochem.* **1997**, *122*, 788.
[15] N. D. Rawlings, *Biochimie* **2010**, *92*, 1463.
[16] M. Laskowski Jr., M. A. Qasim, *Biochim. Biophys. Acta Protein Struct. Mol. Enzymol.* **2000**, *1477*, 324.
[17] W. Bode, R. Huber, *Eur. J. Biochem.* **1992**, *204*, 433.
[18] P. Ascenzi, A. Bocedi, M. Bolognesi, A. Spallarossa, M. Coletta, R. De Cristofaro, E. Menegatti, *Curr. Protein Pept. Sci.* **2003**, *4*, 231.

