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Binding Structure of Elastase Inhibitor Scyptolin A

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Summary

Natural bioactive compounds are of general interest to pharmaceutical research because they may be used as leads in drug development campaigns. Among them, scyptolin A and B from *Scytonema hofmanni* PCC 7110 are known to inhibit porcine pancreatic elastase, which in turn resembles the attractive drug target neutrophil elastase. The crystal structure of scyptolin A as bound to pancreatic elastase was solved at 2.8 Å resolution. The inhibitor occupies the most prominent subsites S1 through S4 of the elastase and prevents a hydrolytic attack by covering the active center with its rigid ring structure. The observed binding structure may help to design potent elastase inhibitors.

Introduction

Cyanobacteria produce numerous secondary metabolites [1, 2]. Early studies focused on the toxic peptide metabolites of freshwater species [3]. Meanwhile, nontoxic compounds such as cyanopeptolins have also become of general interest. Cyanopeptolins are produced by various cyanobacteria species [4] and show remarkable bioactivity [5]. For example, a strong protease inhibitory action was documented for A90720A from *Microchaete loktakensis*, for several micropeptins from *Microcystis aeruginosa* or *M. viridis*, for oscillapeptin from *Oscillatoria agardhii*, and for nostopeptins and nostocyclin from *Nostoc* species [6]. Cyanopeptolins contain a 19-membered ring generated from six amino acid residues by lactone formation between a side chain hydroxyl group of the first residue and the C-terminal carboxylate (Figure 1). The lactonization classifies them as depsipeptides. Associated with the large ring, cyanopeptolins contain a 6-membered ring formed by an Ahp residue, which is a glutamate-γ-aldehyde bound as a hemiaminal to the amide nitrogen of the following amino acid [7]. The double ring is compressed by a *cis*-peptide bond and rigidified by two strong internal hydrogen bonds, as sketched in Figure 1. The N-terminal arm of the cyanopeptolins is variable in length and composition and often N blocked by hydrophobic acids [4].

The cyanopeptolins scyptolin A and B were detected in cultures of the terrestrial cyanobacterium *Scytonema hofmanni* [8]. The N-terminal arm is *N*-butyroyl-Ala-Thr in scyptolin A, whereas scyptolin B has a second *N*-butyroyl-alanyl group esterified to the hydroxyl of Thr2 (Figure 1), resulting in the branched N-terminal arm (*N*-butyroyl-Ala)$_2$Thr [8]. Both compounds contain a 3′-chloro-*N*-methyl-Tyr (cmTyr), the amide methylation of which facilitates the formation of the preceding *cis*-peptide bond. The two scyptolins were shown to inhibit elastase [8], which characteristically degrades elastin forming the basis of flexible connective tissues [9]. An imbalance between such a protease and its natural inhibitors may lead to excessive proteolysis, giving rise to the development of diseases such as pancreatitis, arthritis, emphysema, cystic fibrosis, or psoriasis [10]. Here we report the crystal structure of an elastase in complex with its inhibitor scyptolin A which reveals how the inhibitor uses its four N-terminal amino acid residues to bind specifically to the enzyme and its ring moiety to exclude water and, thus, hydrolysis. These data constitute a lead for the development of antielastase drugs.

Results and Discussion

Interaction of Scyptolin A with Elastase

Purified scyptolin A was added in excess to a solution of porcine pancreatic elastase, and cocrystallization was accomplished by the hanging drop vapor diffusion technique. The resulting space group, P622, is rare. The lacking screw axis explains the small crystal dimension along the 6-fold axis. Various attempts to crystallize a similar complex with scyptolin B failed, although A and B show the same binding behavior in solution. X-ray diffraction data were collected to 2.8 Å resolution (Table 1). An initial (F_o-F_c) electron density map showed the inhibitor binding conformation unambiguously (Figure 2). The electron density at the 3 σ contour level was continuous, excluding a covalent bond with the enzyme.

The two crystallographically independent elastase-inhibitor complexes in the crystal were coupled by a noncrystallographic symmetry relationship across a local 2-fold axis. This improved the model appreciably but diminished all differences between the two complexes. The resulting root-mean-square deviation of all atoms was only 0.025 Å for the peptides and 0.021 Å for the inhibitors. In the following, we therefore refrain from distinguishing between the two copies. The inhibitor molecules contact each other across the local 2-fold axis that, however, does not seem to perturb their conformations. However, this contact excludes an equivalent crystalline complex with scyptolin B because the additional Thr2-Oγ substituent of scyptolin B would clash with the other molecule.

As sketched in Figure 1 and detailed in Figure 3, scyptolin A used its four N-terminal amino acid residues to

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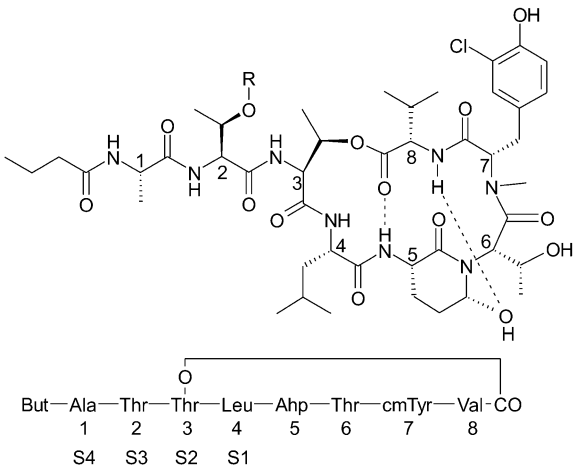

Figure 1. Covalent Structures of the Cyanopeptolins Scyptolin A with R = H and Scyptolin B with R = *N*-Butyroyl-Alanyl

All amino acid residues have the L configuration; the oligopeptide is numbered as usual. Ahp stands for 3-amino-6-hydroxy-2-piperidone. A *cis*-peptide bond is formed between Thr6 and cmTyr (3′-chloro-*N*-methyl-Tyr). The lactone cyclization classifies the molecule as a depsipeptide. The indicated elastase subsites S1 through S4 place the scissile bond between Leu and Ahp.

bind at subsites S1 through S4 of elastase, forming main chain hydrogen bonds like in an antiparallel β sheet. At subsite S1, Leu4-O filled the oxanion hole interacting with the amides of residues 201, 202, and 203. However, hydrolysis did not occur, as the continuous (F_o-F_c) electron density of the 19-membered ring excludes a split of the peptide bond. Further hydrogen bonds were made between Ala1-O of the inhibitor and Arg226-Nε of elastase and between Thr6-Oγ of the inhibitor and Thr44-Oγ. Moreover, scyptolin A accommodates a conspicuous water molecule hydrogen bonded to Thr2-Oγ, Thr3-O, and cmTyr7-O. This water has Gln200-Nε of the enzyme as a fourth partner and is bound in an ideal tetrahedral geometry. This water is likely to further rigidify the large ring. The butyroyl group and the chlorine substituent are in nonpolar environments. The shape and size of scyptolin A allow a snug fit in the active center pocket (Figure 2B).

Inhibition studies of elastase showed an IC_{50} value of 0.16 μM for both scyptolins A and B [8]. Note that the side chain of Thr2 points to the solvent (Figure 2B) so that scyptolin B should fit elastase as well as scyptolin A. The IC_{50} values for trypsin were about 100-fold higher [8], although the scyptolin A binding mode to elastase can be transferred to the structurally related trypsin without causing serious clashes. However, two hydrogen bonds to the N-terminal arm of scyptolin A cannot be formed with trypsin, and Leu4 of the inhibitor cannot bind favorably in the trypsin specificity pocket that is built for Lys or Arg. These differences may explain why scyptolin A is not an efficient trypsin inhibitor.

Table 1. Data Collection, Phasing, and Refinement Statistics[a]

Data Collection	
Resolution range (Å)	30–2.8 (2.86–2.80)
Number of unique reflections	16,453 (999)
Multiplicity	8.3 (8.4)
Completeness	99 (100)
R_{sym-I} (%)	13.3 (39)
I/σ_I	13.6 (5.5)
Molecular Replacement[a]	
Rotation (Rf/σ)	6.52/4.43/4.30
Translation at best rotations (R factor)	0.41
Refinement	
Number of protein atoms	3,644
Number of inhibitor atoms	136
Number of water molecules	105
Average B factor (Å^2)	24.5
R_{cryst}	0.209
R_{free} (10% test set)	0.258
Rmsd bond lengths/bond angles	0.007 Å/1.35°
Ramachandran plot favored/allowed (%)[b]	83/16

The space group was P622 with unit cell parameters a = b = 155.9 Å and c = 91.0 Å, two elastase molecules per asymmetric unit, and a solvent content of 61%. The data were collected at 100 K using Cu Kα radiation. Numbers in parentheses refer to the last shells. All data were used without σ cutoff.

[a] The three best solutions of the rotation function of MOLREP [20] are given. The first two were related to each other by a local 2-fold axis representing the noncrystallographic symmetry of the two molecules in the asymmetric unit.

[b] Residues His75 and Tyr178 were in the generously allowed region [27].

Comparison with Other Inhibitors

The elastase-scyptolin A complex showed that subsites S1 through S4 are most important, whereas subsites S1′, S2′, etc. at the other side of the scissile bond cannot be easily defined. The same difference was reported for several other elastase-inhibitor complexes [11–15]. Among them, binding studies with an elastase-inhibiting casein peptide [12, 13] revealed that only subsites S1 through S4 were occupied and that the substrate had formed an ester with the enzyme (Figure 3). This structure also contained a water molecule in a suitable location for ester hydrolysis [13], which is depicted in Figure 3. This "hydrolytic water" is at the same position as the Ahp residue within the 19-membered ring of scyptolin A (Figure 3), demonstrating that the macrocycle of the scyptolins occupies the crucial part of the active center pocket, thus preventing hydrolysis. Further elastase inhibitor complex structures were reported for the *Streptomyces* peptide derivative FR901277 [14] and for the designed inhibitor FR136706 [15]. The latter two inhibitors also filled the active center pocket well, albeit with chemical structures that deviated appreciably from scyptolin A, opening a different approach to elastase inhibition.

The binding structure of scyptolin A to elastase should be compared with that of cyanopeptolin A90720A to the structurally related bovine pancreatic trypsin [16]. A superposition of the inhibitors showed nearly identical conformations of the 19-membered rings, including the Ahp residues and the two internal hydrogen bonds, although residues Leu4, Thr6, and cmTyr7 of the scyptolins are exchanged for Arg, Leu, and mTyr of A90720A, respectively. The Leu to Arg exchange before the scissile bond of course reflects the specificity difference

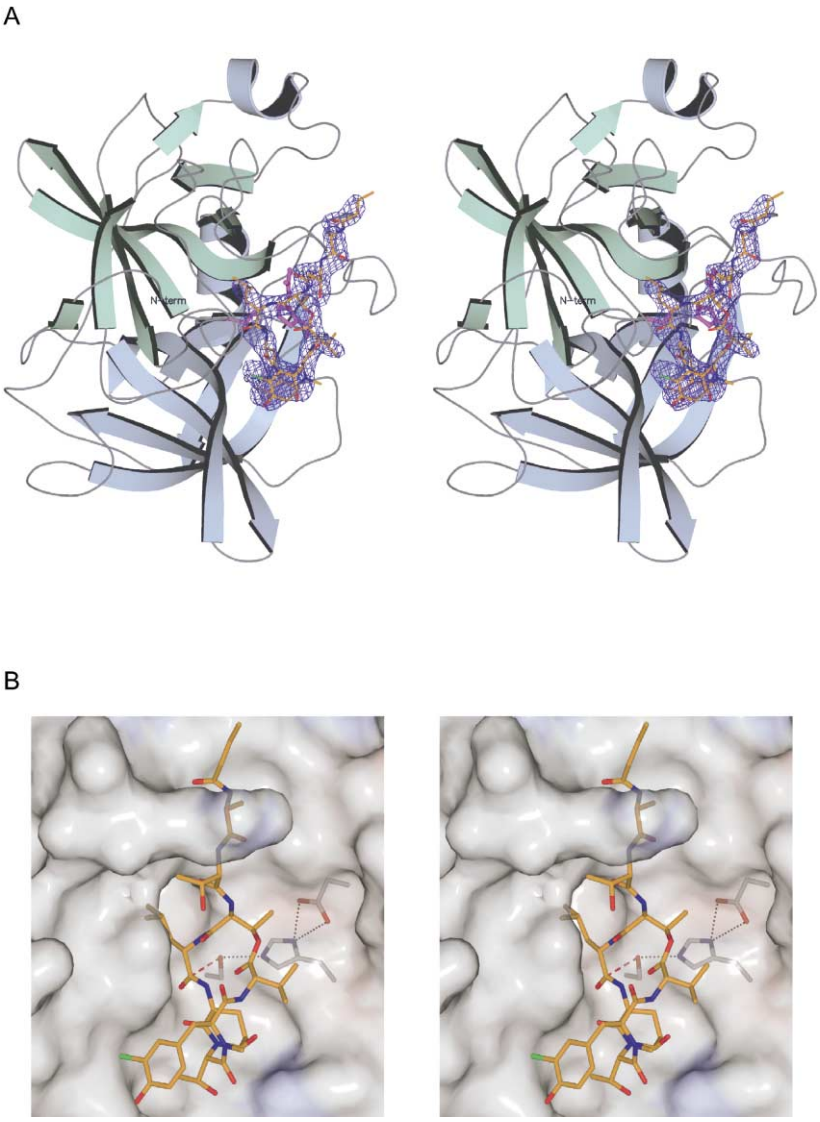

Figure 2. Stereo Views of Scyptolin A Binding to Porcine Pancreatic Elastase

(A) Ribbon plot of elastase with the initial (F_o-F_c) electron density map of scyptolin A at a contour level of 3 σ. The final model of scyptolin A is drawn into the density. The side chains of the catalytic triad of elastase are given for reference (pink).

(B) Stereo view of scyptolin A bound to elastase represented by its molecular surface. The catalytic triad beneath the surface is shown; the putative nucleophilic attack of the serine is indicated by a dashed red line. The inhibitor with its rigid cyclic peptide structure sits snugly in the active center pocket.

between elastase and trypsin. The N-terminal arm of A90720A consists of a D-Leu that is *N* blocked by (*R*)-glycerate-3-sulfate. In both instances, the 19-membered ring remained intact as no ester was formed with the catalytic serine of the enzyme. In conclusion, the two cyanopeptolins show closely related ring structures and a similar inhibition geometry, although at different enzymes [16].

Significance

Several cyanopeptolins are known to inhibit serine proteases, some of which fulfill important functions and thus constitute potential targets for pharmaceutical research. The serine protease elastase is required for the degradation and/or reorganization of elastic fibers in vertebrate tissues, such as skin, blood vessels, and lungs. Since scyptolin A inhibits elastase at submicromolar concentrations [8], it constitutes a lead for the design of drugs counteracting elastase-related diseases such as emphysema. A comparison with cyanopeptoline A90720A binding to trypsin [16] revealed closely related conformations of the 19-membered rings and similar binding interfaces. In contrast to these two cyanopeptolins, synthetic inhibitors use a broad range of binding modes [11].

Although little is known about the ecological impact of the cyanopeptolins, it has been proposed that some metabolites of marine cyanobacteria act as feeding deterrents [17]. These cyanobacteria commonly pro-

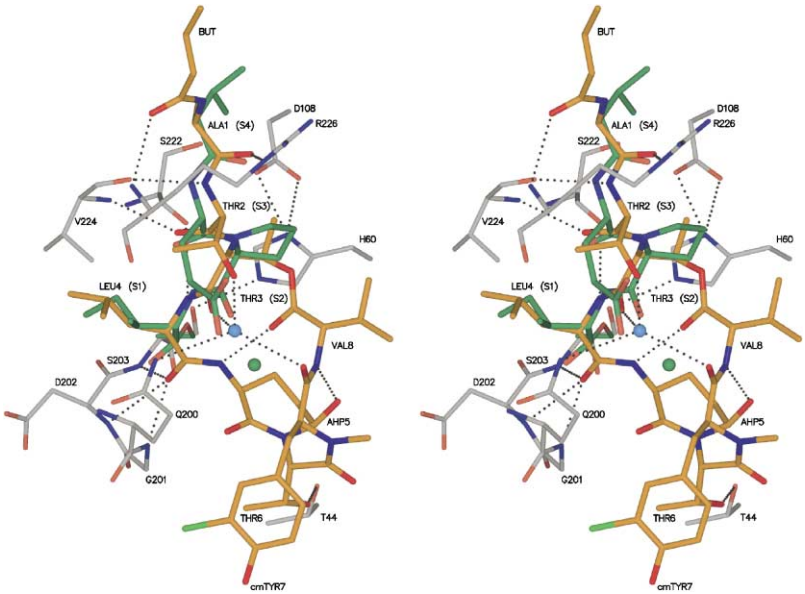

Figure 3. Stereo View of the Elastase Binding Structure of Scyptolin A Superimposed with that of the Elastase-Inhibiting Peptide Val-Glu-Pro-Ile

Val-Glu-Pro-Ile is derived from β-casein [13] and forms an ester bond to the catalytic Ser203 (green). The casein peptide structure [13] reveals the four subsites S1 through S4 that bind the substrate, forming an antiparallel β sheet interface. It also contains a water molecule at a position suitable for hydrolysis (green). The four N-terminal amino acid residues of scyptolin A occupy subsites S4 through S1 of elastase. The internal water molecule stabilizing the ring structure of scyptolin A is shown.

duce a broad spectrum of peptide metabolites and grow in mixed assemblages with other organisms, usually bacteria or diatoms [2]. The adaptive advantage of numerous rather than a single defensive compound is obvious. The capability to change the composition of its natural product spectrum is probably essential for the survival of cyanobacteria in a complex community [18].

Experimental Procedures

Scyptolin A was isolated from axenic cultures of *S. hofmanni*, purified as described [8], and dissolved in methanol at 33 μg/ml. Lyophilized porcine pancreatic elastase (no. 20929.01, Serva) was dissolved in 10 mM sodium acetate buffer (pH 5.0) at a concentration of approximately 18 mg/ml and incubated 30 min with a 1.5 molar excess of the scyptolin solution. Using the hanging drop set-up, 10 μl drops containing equal volumes of the elastase-scyptolin solution and of the reservoir solution of 0.42 M sodium sulfate were equilibrated against 500 μl reservoir solution. Crystals forming hexagonal plates of sizes up to 200 × 200 × 20 μm^3 grew within 2–3 days. The crystals were equilibrated for about 2 min in a solution of 50% glycerol containing 0.025 M sodium sulfate and flash frozen to 100 K.

Data were collected to 2.8 Å resolution using an image plate (model 300, Marresearch) with Cu Kα radiation from a rotating anode (model RU200B, Rigaku). Data were processed with programs XDS and XSCALE [19]. Phases were obtained by molecular replacement with MOLREP [20]. The search model was an elastase structure taken from the Protein Data Bank (ID code 1ELB) without substrate and water molecules. After rigid body refinement with noncrystallographic symmetry restraints using program CNS [21], model bias was removed by a simulated annealing run starting at a temperature of 2500 K. An initial (F_o-F_c) difference electron map was then calculated from the annealed model. Coordinates for an energy-minimized conformation of the inhibitor and the corresponding topology and parameter files were generated with program PRODRG [22]. Structural refinement was performed with program CNS [21]; manual adjustments were carried out with program O [23]. Due to the limited resolution, only two groups (main chain and side chain) were used in the B-factor refinement. Figures were produced with the programs MOLSCRIPT [24], POVScript [25], and RASTER3D [26].

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Accession Numbers

The coordinates and structure factors have been deposited in the Protein Data Bank under ID code 1OKX.