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Binding structure of the leucine aminopeptidase inhibitor microginin FR1

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Abstract Natural bioactive compounds are of general interest for pharmaceutical research because they may serve as leads in drug development campaigns. Among them, microginins are linear peptides known to inhibit various exopeptidases. The crystal structure of microginin FR1 from *Microcystis* sp. bound to bovine lens leucine aminopeptidase was established at 1.73 Å resolution. The observed binding structure could be beneficial for the design of potent aminopeptidase inhibitors.
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1. Introduction

Water blooms in eutrophic lakes are often caused by cyanobacteria [1]. Field populations of the common bloom-forming genus *Microcystis* can be toxic because some strains produce microcystins, which are hepatotoxic, promote tumors and affect the development of fish and amphibians [2–4]. In addition, cyanobacteria produce non-toxic peptides, some of which show remarkable bioactivities and have therefore attracted the attention of the pharmaceutical industry [5,6]. Of particular interest are phosphatase inhibitors [7] and selective protease inhibitors, as for example the elastase inhibitor scyptolin A [8].

Among these peptides are microginins (Fig. 1), the first of which was isolated from *Microcystis aeruginosa* (NIES-100) [9]. Microginins contain the lipidic β-amino acid Ahda (3-amino-2-hydroxy-decanoic acid) and inhibit the angiotensin-converting enzyme (ACE) [9] as well as aminopeptidases but do not affect papain, trypsin, chymotrypsin or elastase [10]. Related to the microginins are the well-known inhibitors bestatin [11] and amastatin [12] produced by *Streptomyces*. Both of them inhibit aminopeptidases [13]; bestatin causes a reduction of the HIV-infection rate [14]. Here we report the binding structure of microginin FR1 that was extracted from a water bloom in a local lake [15] to a leucine aminopeptidase (LAP).

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Abbreviations: ACE, angiotensin-converting enzyme; Ahda, (2*S*, 3*R*)-3-amino-2-hydroxy-decanoic-acid; LAP, leucine aminopeptidase

2. Materials and methods

Microginin FR1 was isolated from water bloom material in Lake Waltershofen near Freiburg and the IC_{50} value for bovine lens LAP was determined as described [16]. The enzyme was isolated from calf lenses as described [17]. The calf eyes were received 5 h after slaughtering and stored on wet ice. The isolated enzyme ran through an additional gel permeation column (Superdex-200 26/60, Amersham) equilibrated with buffer A (50 mM Tris–HCl pH 7.8, 50 μM $ZnSO_4$ and 200 mM NaCl). LAP-containing fractions were pooled and concentrated to 7 mg/ml in buffer A. Microginin FR1 dissolved in DMSO was added at a concentration of 5 mM to the enzyme solution, incubated for 30 min at 37 °C and centrifuged. Using the hanging drop set-up, 10 μl of the enzyme-plus-inhibitor solution was equilibrated against buffer B (50 mM Tris–HCl pH 7.8, 50 μM $ZnSO_4$, 50% (v/v) 2-methyl-2,4-pentanediol) [18]. The crystals were flash-frozen to 100 K without any further cryo-protectant.

X-ray diffraction data were collected at beamline BL14-1 of BESSY (Berlin) and processed using the programs XDS and XSCALE [19]. The structure of bovine lens LAP (Protein Data Bank code **1BLL**) [18] was used as a starting model for structure determination. After rigid body refinement with the program REFMAC5 [20], model bias was removed by a simulated annealing run starting at a temperature of 2500 K with CNS [21]. An initial map was calculated using RESOLVE [22]. After manual adjustments with the program COOT [23], the model was refined in REFMAC5 and the inhibitor was placed in the resulting (F_o–F_c) difference electron density map. An energy-minimized conformation of the inhibitor and the corresponding dictionary files were generated with PRODRG [24]. Water molecules were placed using COOT and the refinement was finalized with REFMAC5. The coordinates and structure factors are deposited in the Protein Data Bank code **2J9A**.

3. Results and discussion

Purified microginin FR1 was added in excess to a solution of LAP from bovine lens, and cocrystals were obtained by the hanging drop technique. X-ray diffraction data were collected to 1.73 Å resolution and the structure was determined (Table 1). An initial (F_o–F_c)-electron density map showed the inhibitor binding conformation of the first three residues unambiguously. After refinement, the remaining two residues were also placed unambiguously although with higher *B*-factors (Fig. 2A).

Bovine lens LAP is a homohexamer with D_3 symmetry (Fig. 2B). Two large contacts around the twofold axes are formed by the N- and C-terminal domains in different subunit pairings, tightly interconnecting the hexamer. The C-terminal domains form a further contact around the threefold axis. The active centers are on the inside of the hexamer lining a large central cavity. They are shielded by the N-terminal domains but accessible through six channels with a width appropriate for an extended peptide chain (Fig. 2C). With such a topology, the

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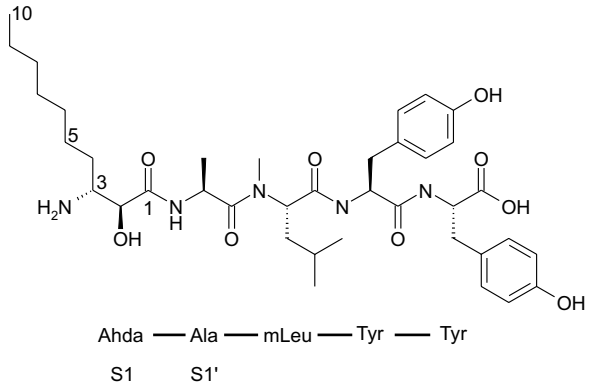

Fig. 1. Covalent structure of the pentapeptide microginin FR1 with its characteristic Ahda residue [15]. The related dipeptide bestatin [11] and tetrapeptide amastatin [12] carry a modified Ahda as the first residue. In bestatin and amastatin the atoms C5–C10 of Ahda are replaced by phenyl and isopropyl, respectively. The other residues of bestatin and amastatin are -Leu and -Val-Val-Asp, respectively. All amino acid residues have the L-configuration. The position of the scissile bond of processed peptides is indicated by the assigned subsites S1 and S1′.

enzyme can only cleave unfolded peptides, because folded peptides or proteins cannot enter the central cavity.

The Ahda-Ala part of microginin FR1 fits snuggly into the active center pocket (Fig. 3), binding with full occupancy as indicated by the crystallographic *B*-factors matching those of the environment. The decanoic acid moiety of Ahda is accommodated in a spacious pocket extension formed by Leu269, Met270, Asp365, Ala451, Met454 and Thr455. This extension is for the most part nonpolar but contains Asp365 at one side, suggesting that a positive charge at this part of the inhibitor would increase the binding strength. The amino group of Ahda interacts with one of the two zinc ions at the active center (Zn1) as well as with Thr359-O, while the hydroxyl group of Ahda interacts with Zn2 and with a water molecule tightly bound to the backbone amides of Glu334 and Gly335. The 1-carbonyl of Ahda forms a hydrogen bond to Lys262-Nε. The alanine of microginin FR1 forms hydrogen bonds with Leu360-O and Gly362-N reminiscent of a piece of parallel β-sheet. Ahda and alanine occupy subsites S1 and S1′ (Fig. 1), thus covering the active center of the LAP.

Instead of the carbonate or the three water molecules found in other bovine lens LAP structures [25,26], we observed a chloride ion bound to Arg366 close to the metal ion Zn2 (Fig. 3A). Since a water molecule placed at this position caused positive difference electron density and since the observed density is at the level of several sulfur atoms, a chloride ion from the crystallization buffer is the most likely interpretation.

The three C-terminal residues of the inhibitor are involved in less clearly defined binding patterns. The third residue *N*-methyl-leucine binds with its carbonyl oxygen through a water network to Arg336 and Ala363-O. The remaining two tyrosines of microginin FR1 show rather high *B*-factors (Fig. 3A). The first tyrosine binds with its hydroxyl through Arg425′ to Asp365. The hydroxyl of the second tyrosine forms hydrogen bonds through water molecules and directly to the backbone of residues 423 and 424. Despite their high *B*-factors, the removal of these tyrosines weaken the inhibitory capacity appreciably [16]. Other inhibitor complexes with bovine lens LAP are known [25].

The cyanobacterial peptide microginin FR1 represents a large group of known microginins characterized by the N-terminal residue Ahda [9,10,27,28]. The IC_{50} values of microginin FR1 for bovine lens cytosolic LAP (EC 3.4.11.1) was determined as 1.3 μM, which is lower than the value 16 μM for ACE [15]. The IC_{50} values of the other microginins for ACE are in the range of 10 μM or above [9,16,28]. Stronger inhibition was observed for the microsomal LAP (EC 3.4.11.2). Microginin FR1 shows an IC_{50} value of 6 nM with the porcine kidney microsomal LAP, while other microginins range from 16 nM to around 10 μM [9,10,16,28]. The related inhibitors bestatin and amastatin show IC_{50} values in the micromolar range for cytosolic and microsomal LAP [13,29].

The inhibited enzymes cytosolic LAP, microsomal LAP and ACE are all exopeptidases with one or two zinc ions in the active center. However, their structures are completely different [30–32]. All discussed inhibitors have Ahda-related first residues (Fig. 1), which bind to the metal centers of cytosolic and microsomal LAP as shown in Fig. 3 and elsewhere [18,30,32,33]. The reported binding structure to cytosolic

Table 1
Structure determination[a]

Data collection	
Resolution (Å)	35 – 1.73 (1.80 – 1.73)
Unique reflections	62705 (6822)
Multiplicity	6.0 (5.4)
Completeness (%)	99.4 (98.1)
R_{sym} (%)	6.9 (47)
I/σ_I	19.0 (3.5)
Wilson *B*-factor (Å^2)	23.1
Refinement	
Protein atoms	3921
Solvent atoms	539
Microginin FR1 atoms	52
R_{cryst}/R_{free} (5% test set)	14.5/17.4
Average *B*-factor (Å^2)	19
Rmsd bond lengths (Å)/bond angles (°)	0.019/1.596
Ramachandran:favored/allowed (%)	93.1/6.9

[a]The data were collected at a wavelength of 0.9184 Å at BESSY (Berlin). The space group was $P6_322$ with unit cell parameters $a = b = 130.0$ Å, $c = 120.8$ Å and one subunit in the asymmetric unit.

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