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Isolation, crystallization and preliminary X-ray diffraction analysis of L-amino-acid oxidase from *Vipera ammodytes ammodytes* venom

Dessislava Georgieva,[a,b] Anna Kardas,[a] Friedrich Buck,[c] Markus Perbandt[a] and Christian Betzel[a]*

[a]Institute of Biochemistry and Molecular Biology, University of Hamburg, c/o DESY, Gebäude 22a, Notkestrasse 85, 22603 Hamburg, Germany, [b]Institute of Organic Chemistry, Bulgarian Academy of Sciences, Akad. G. Bonchev bl. 9, 1113 Sofia, Bulgaria, and [c]Institute of Clinical Chemistry, University Medical Center Hamburg – Eppendorf, Martinistrasse 52, 20246 Hamburg, Germany

Correspondence e-mail: betzel@unisgi1.desy.de

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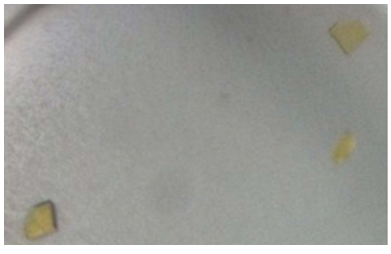

L-Amino-acid oxidase from the venom of *Vipera ammodytes ammodytes*, the most venomous snake in Europe, was isolated and crystallized using the sitting-drop vapour-diffusion method. The solution conditions under which the protein sample was monodisperse were optimized using dynamic light scattering prior to crystallization. The crystals belonged to space group *C*2, with unit-cell parameters $a = 198.37$, $b = 96.38$, $c = 109.11$ Å, $\beta = 92.56^\circ$. Initial diffraction data were collected to 2.6 Å resolution. The calculated Matthews coefficient is approximately 2.6 Å^3 Da^{-1} assuming the presence of four molecules in the asymmetric unit.

1. Introduction

L-Amino-acid oxidase (LAAO; EC 1.4.3.2) catalyzes the oxidative deamination of a number of L-amino acids to their respective imino acids with concomitant hydrolysis to α-keto acids, together with the production of ammonia and hydrogen peroxide,

$$R\mathrm{CHNH_2COOH} + \mathrm{O_2} + \mathrm{H_2O} \rightarrow R\mathrm{COCOOH} + \mathrm{NH_3} + \mathrm{H_2O_2}.$$

The mechanism of dehydrogenation by LAAO includes hydride transfer (Faust *et al.*, 2007). The enzyme contains noncovalently bound flavin adenine dinucleotide (FAD) as a cofactor (Zeller & Maritz, 1944). LAAOs have been found in various species, both prokaryotic and eukaryotic (Niedermann & Lerch, 1991; Nishizawa *et al.*, 2005; Vallon *et al.*, 1993); they constitute up to 30% of the total protein in snake venoms and contribute significantly to their toxicity. Snake-venom L-amino-acid oxidases (SV-LAAOs) are of great interest because they play a role in toxin-induced apoptosis, affecting platelets and exerting haemorrhagic and cytotoxic effects (Suhr & Kim, 1996; Du & Clemetson, 2002 and references therein). These enzymes are multifunctional proteins that also exhibit antibacterial and anti-HIV activities (Stiles *et al.*, 1991; Moustafa *et al.*, 2006). It is believed that the H_2O_2 produced by SV-LAAOs is involved in apoptosis, a process that leads to cell death. However, there are indications that the enzyme itself can be related, at least in part, to this effect (Du & Clemetson, 2002). LAAO from *Agkistrodon acutus* venom induces cell apoptosis *via* the Fas pathway (Zhang & Cui, 2007). *Bothrops moojeni* LAAO kills *Leishmania* spp. promastigotes and can be used in the treatment not only of leishmaniasis but also of other intracellular parasites (Tempone *et al.*, 2001). *A. blomhoffii* venom LAAO causes pneumorrhagia, pulmonary and cardiac oedema and bleeding in BALB/c mice as well as liver-cell necrosis (Wei *et al.*, 2007). The enzyme induces lesions in the lungs and liver. The crystal structure of LAAO from *Calloselasma rhodostoma* has been determined in the presence of ligands (Pawelek *et al.*, 2000; Moustafa *et al.*, 2006).

In a previous paper (Georgieva *et al.*, 2008), we described the venom proteomics of *Vipera ammodytes ammodytes* and *V. ammodytes meridionalis*, the most poisonous reptiles in Europe. LAAO is one of the major toxin components in the venoms of both snakes. The *V. ammodytes ammodytes* venom contains five isoforms of the oxidase and that of the *V. ammodytes meridionalis* four isoenzymes. Here, we describe the purification, crystallization and preliminary X-ray analysis of LAAO from the venom of *V. ammodytes ammodytes*, named VAA-LAAO I. The enzyme we isolated is a novel

snake-venom LAAO with a different primary structure which suggests possible differences in its other levels of structural organization and in its specificity.

2. Materials and methods

2.1. Purification

V. ammodytes ammodytes specimens were captured in northwest Bulgaria. Snakes of both sexes were milked and the venom was lyophilized and stored at 277 K until used. 100 mg crude venom was dissolved in 5 ml starting buffer (50 m*M* acetic acid pH 5.0) and fractionated by FPLC as described previously (Georgieva *et al.*, 2008). In brief, a small amount of precipitate was removed by centrifugation and the clear supernatant was loaded onto a MonoS (Amersham-Pharmacia, Freiburg) column pre-equilibrated with the starting buffer and then eluted with a linear gradient from 0 to 1 *M* NaCl in the same buffer. Five LAAO isoforms were found in the venom of *V. ammodytes ammodytes* which differed in their isoelectric points and/or molecular weight. Fractions containing LAAO activity were pooled, dialyzed against 20 m*M* Tris–HCl pH 8.0 and subsequently loaded onto a MonoQ (Amersham-Pharmacia, Freiburg) column and eluted with a three-step linear gradient from 0 to 1 *M* NaCl in the same buffer (Fig. 1). After rechromatography on the same column, the enzyme was eluted as a single peak. VAA-LAAO I was dialyzed against distilled water and concentrated to 17 mg ml^{-1}. The protein was homogeneous by 12% SDS–PAGE and dynamic light scattering using a RiNA GmbH DLS system (Spectroscatterer 201; Berlin, Germany) with a He–Ne laser providing 690 nm light and an output power in the range 10–50 mW. An autopiloted run with 20 measurements every 30 s was used. The enzyme was stored at 277 K and LAAO activity was measured as described by Wellner & Meister (1961).

2.2. MS/MS analysis of internal peptides

The protein sample was subjected to gel electrophoresis and the gel was stained with Coomassie Brilliant Blue. The bands of interest were cut from the polyacrylamide gels and the protein was reduced with 10 m*M* DTT at 329 K for 30 min. Cysteine residues were modified with 55 m*M* iodoacetamide at room temperature for 20 min. The protein was digested in the gel by trypsin in 50 m*M* ammonium carbonate at 310 K overnight. The protease concentration was 5 ng µl^{-1}. The peptides were eluted with 50% acetonitrile/5% formic acid and concentrated by vacuum centrifugation. The samples were redissolved in 5% methanol/5% formic acid, desalted on a C18

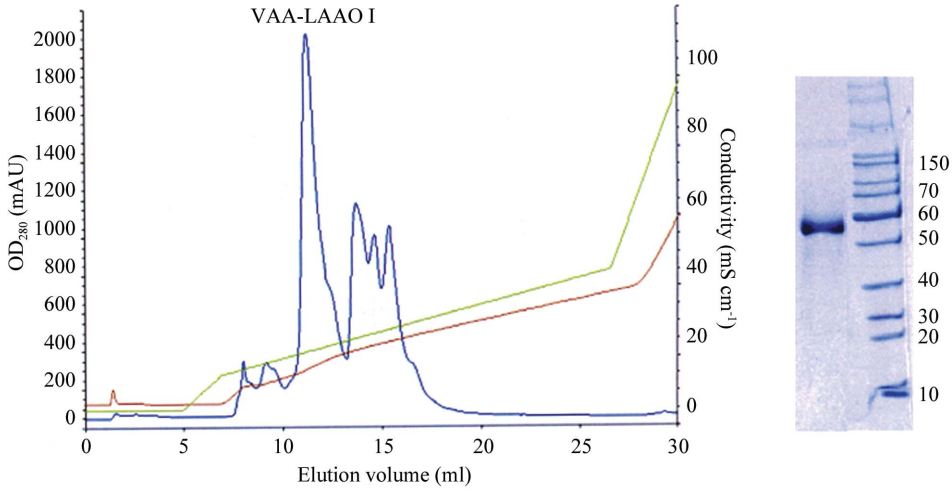

Figure 1
(*a*) Fractionation of *V. ammodytes ammodytes* venom L-amino-acid oxidases by FPLC using a MonoQ column. The major peak contains VAA-LAAO I. The column was eluted with a three-step linear gradient from 0 to 1 *M* NaCl in 20 m*M* Tris–HCl pH 8.0. (*b*) SDS–PAGE of VAA-LAAO I. The right-hand lane contains molecular-weight markers (labelled in kDa).

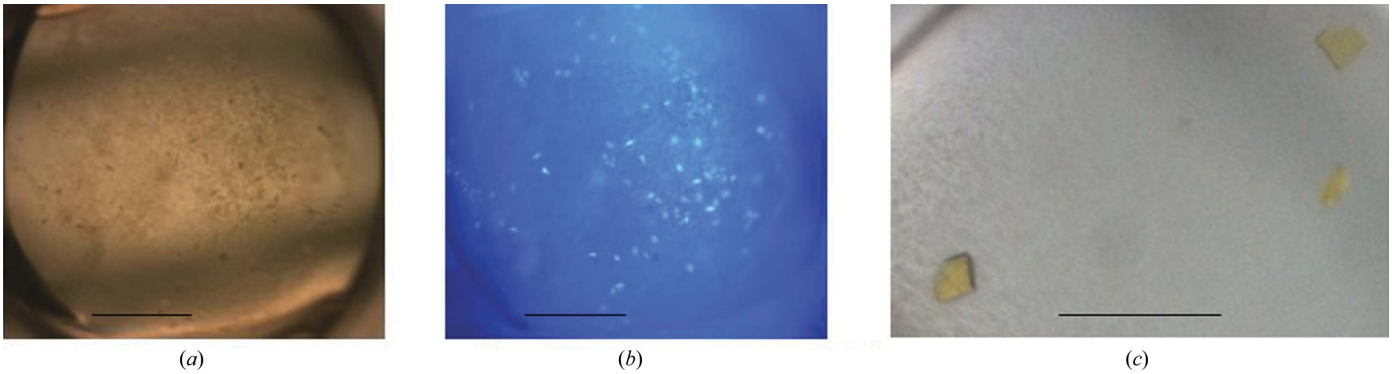

Figure 2
Crystals of *V. ammodytes ammodytes* venom L-amino-acid oxidase. (*a*) Small crystals obtained at a protein concentration of 17 mg ml^{-1} using 20%(*w*/*v*) PEG 3350, 0.05 *M* zinc acetate. (*b*) The same crystals illuminated with UV light applying a Dual DUVI 201 light source (RiNA GmbH, Berlin, Germany). (*c*) Single crystals obtained at a protein concentration of 10 mg ml^{-1} using 26%(*w*/*v*) PEG 3350, 0.04 *M* zinc acetate.

Table 1
MS/MS-derived sequences of peptides obtained after tryptic hydrolysis of L-amino-acid oxidase from *V. ammodytes ammodytes* venom.

Homology with related proteins was found using a *BLAST* search. M*, oxidized methionine; C**, iodoacetamide-modified cysteine.

Peptide-ion *m*/*z*	*z*	MS/MS-derived sequence	Homology with related proteins from	Accession code
750.79	2	EDDYEEFLEIAK	*Macrovipera lebetina*	P81375
			Vipera berus berus	P0C2D7
826.34	2	NVEEGWYANLGPM*R	*Trimeresurus steijnegeri*	Q6WP39
1224.57	2	FLGKLNEFVQETDNGWYFIK	*Daboia russellii siamensis*	Q4F867
916.69	3	FDEIVGGM*DELPTSM*YKAIEESVR	*Daboia russellii siamensis*	Q4F867
645.77	2	TFC**YPSM*IQK	*Trimeresurus steijnegeri*	Q6WP39
			Gloydius blomhoffi	Q90W54
			Gloydius halys	Q9STF1
1525.68/1017.45	2/3	YAM*GAITTFTPYQFQHFSEALTAPEGR	*Daboia russellii siamensis*	Q4F867
747.67	3	IFFAGEYTANAHGWIDSTIK	*Daboia russellii siamensis*	Q4F867
			Vipera berus berus	P0C2D7

μZipTip (Millipore), eluted with 1 μl 60% methanol/5% formic acid and analysed by nano-electrospray mass spectrometry using a QTOF II instrument (Micromass, Manchester, England). The obtained MS/MS data were used for homology searches in the Swiss-Prot and NCBI data banks using the *BLAST* algorithm. The complete amino-acid sequence of VAA-LAAO I is under determination. Preliminary data showed sufficient homology with the related enzyme from *Calloselasma rhodostoma* (PDB code 2iid), the three-dimensional structure of which will be used for molecular replacement and model building.

2.3. Crystallization

Crystallization conditions were screened by the sitting-drop vapour-diffusion technique using Crystal Screen and Index Screen Kits (Hampton Research) at 293 K with the robotic Honeybee 961 system (Genomic Solutions). Drops were prepared by mixing 400 nl reservoir solution and 400 nl solution consisting of 17 mg ml^{-1} protein in 50 m*M* Tris pH 8.0 (Fig. 2*a*). The first crystals appeared after 2 d from 20%(*w*/*v*) PEG 3350 and 0.05 *M* zinc acetate. These conditions were further optimized and single crystals suitable for X-ray analysis were obtained as follows: 1 μl protein solution with concentration 10 mg ml^{-1} was mixed with 1 μl of a precipitant solution consisting of 16%(*w*/*v*) PEG 3350 and 0.04 *M* zinc acetate. The volume of the reservoir solution was 1 ml (Fig. 2*c*). The drops were equilibrated at 293 K and crystals suitable for X-ray diffraction were obtained within 2 d. The crystals were illuminated with a special white-light/UV-light source to confirm that they were protein crystals using the tryptophan-based fluorescence as an indicator (Fig. 2*b*).

2.4. Data collection and processing

Diffraction data were collected to 2.6 Å resolution from a flash-frozen crystal at 100 K using synchrotron radiation (λ = 0.806 Å) on the consortium beamline X13 at HASYLAB/DESY, Hamburg, Germany. Crystals were picked out with a loop and soaked in a cryoprotectant solution consisting of 16%(*w*/*v*) PEG 3350, 0.04 *M* zinc acetate and 15%(*v*/*v*) glycerol. A total of 180 diffraction images

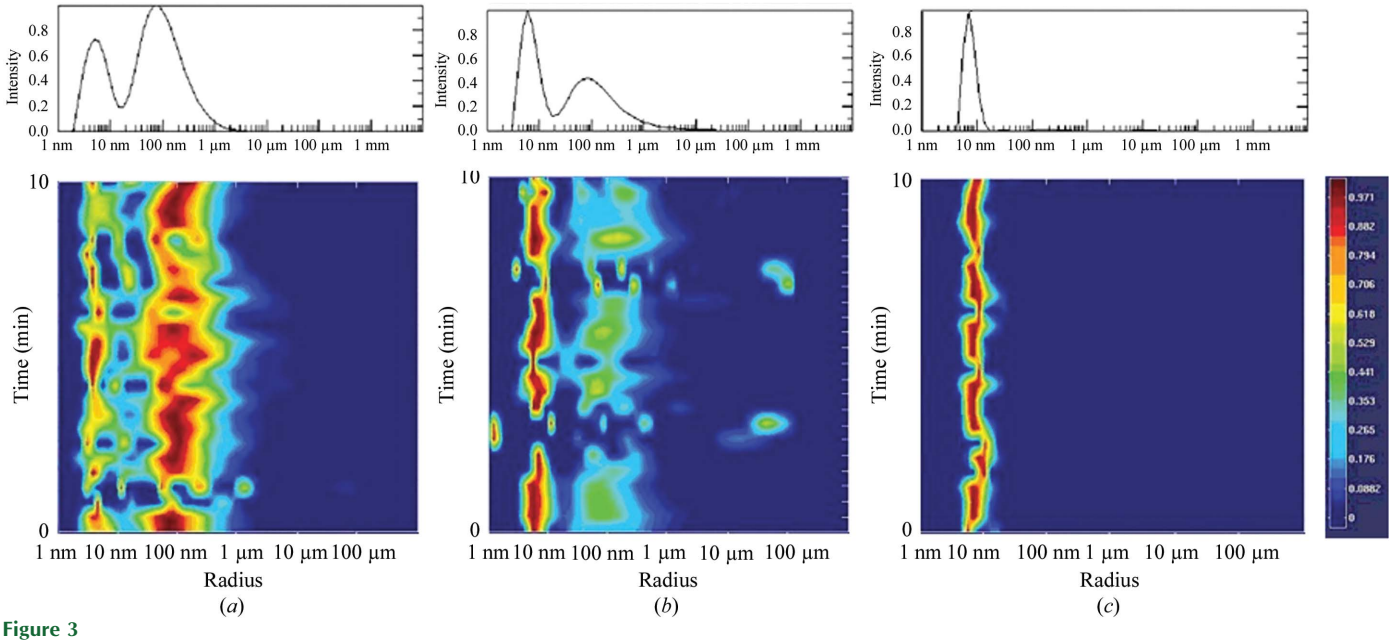

Figure 3
Optimization of the crystallization conditions of *V. ammodytes ammodytes* venom L-amino-acid oxidase using dynamic light scattering. (*a*) Formation of high-molecular-weight aggregates precluding crystallization at a protein concentration of 13 mg ml^{-1} in water. (*b*) Nonhomogeneous solution at a protein concentration of 8 mg ml^{-1} in 50 m*M* Tris buffer pH 7.5; the enzyme does not crystallize under these conditions owing to the formation of protein oligomers. (*c*) Homogeneous solution at a protein concentration of 17 mg ml^{-1} in 50 m*M* Tris pH 8.0; crystals suitable for X-ray analysis were obtained using this protein solution. The two-dimensional dynamic light-scattering graphics showing the time-dependence of the particle radii are shown in the lower part of each figure. The colour indicates the relative quantity of each radius fraction: red is the highest and blue lowest.

Table 2
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Space group	*C*2
Unit-cell parameters (Å, °)	a = 198.4, b = 96.4, c = 109.1, β = 92.56
V_M (Å^3 Da^{-1})	2.6
Wavelength (Å)	0.806
Resolution (Å)	20–2.6
Completeness (%)	99.9 (97.6)
Average $I/\sigma(I)$	13.0 (3.0)
No. of images	180
Raw measurements used	331108
Unique reflections	63105
R_{merge}†	0.175 (0.197)
Subunits per asymmetric unit	4

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith intensity measurement of reflection hkl, including symmetry-related reflections, and $\langle I(hkl)\rangle$ is its average.

were collected with a 1° rotation between images. The programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997) were used for data processing and analysis.

3. Results and discussion

A novel LAAO from *V. ammodytes ammodytes* venom (VAA-LAAO I) was purified to electrophoretic homogeneity. A molecular weight of 55 kDa was determined by SDS–PAGE under reducing and nonreducing conditions. Internal peptide amino-acid sequences obtained after MS/MS analysis of tryptic peptides showed a homology to other snake-venom LAAOs (Table 1). The solution conditions under which the protein sample was monodisperse were optimized using dynamic light scattering prior to crystallization. Fig. 3 shows some representative results demonstrating the application of DLS for this purpose. The upper parts of the figure demonstrate single measurements of the particle-size distribution 5 min after preparation of the solution. The lower parts show that the particles preserve their size over time. The presence of high-molecular-weight aggregates in the protein solution precludes the crystallization process. At a protein concentration of 13 mg ml^{-1} in water, two types of particles with hydrodynamic radii (R_h) of 4.8 and 64.6 nm were observed (Fig. 3*a*). No crystals were obtained under these conditions, most probably owing to the formation of protein aggregates. In the presence of 50 m*M* Tris buffer pH 7.5, particles with R_h of 6.3 nm and oligomers of 77.5 nm were observed (Fig. 3*b*). Again, high-molecular-weight protein aggregates prevented crystallization. A homogeneous solution was obtained at an LAAO concentration of 17 mg ml^{-1} in 50 m*M* Tris buffer pH 8.0. Under these conditions only particles of 7.44 nm were observed (Fig. 2*c*) and crystals suitable for X-ray analysis were grown as described above (Fig. 2). The crystal lattice belonged to space group *C*2. Autoindexing yielded unit-cell parameters a = 198.4, b = 96.4, c = 109.1 Å, β = 92.56°. Packing-parameter calculations based on the molecular weight indicated the presence of four LAAO subunits in the asymmetric unit, but both three and five molecules are also possible. This corresponds to a typical Matthews coefficient V_M of 2.6 Å^3 Da^{-1} and a solvent content of approximately 53%. The data-collection parameters and statistics are summarized in Table 2. Initial calculations to solve the phase problem by molecular replacement are in progress, as well as attempts to further optimize the crystals for high-resolution data collection.

SV-LAAOs contribute considerably to venom toxicity. Current knowledge of the mechanisms of their pharmacological activities is insufficient and further studies need to be performed in order to elaborate adequate therapeutical methods. Three-dimensional structure determination will allow the development of inhibitors that are suitable for pharmaceutical purposes.

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