Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Crystallization and preliminary X-ray analysis of a bacterial L-amino-acid oxidase from *Rhodococcus opacus*

Annette Faust,[a] Birgit Geueke,[b] Karsten Niefind,[a] Werner Hummel[c] and Dietmar Schomburg[a]*

[a]Institute of Biochemistry, University of Cologne, Zuelpicher Strasse 47, 50674 Cologne, Germany, [b]Swiss Federal Institute of Environmental Science and Technology (EAWAG), Ueberlandstrasse 133, 8600 Duebendorf, Switzerland, and [c]Institute of Molecular Enzyme Technology, Heinrich-Heine University of Duesseldorf at Research Centre Juelich, 52426 Juelich, Germany

Correspondence e-mail: d.schomburg@uni-koeln.de

Received 23 January 2006
Accepted 15 February 2006

L-Amino-acid oxidases (EC 1.4.3.2) catalyse the stereospecific oxidative deamination of an L-amino-acid substrate to an α-keto acid with the production of ammonia and hydrogen peroxide. In this study, the crystallization and preliminary X-ray analysis of a bacterial L-amino-acid oxidase from *Rhodococcus opacus* (RoLAAO) is described. RoLAAO is a dimeric protein consisting of two identical subunits of 489 amino acids with a calculated molecular weight of 54.2 kDa and a non-covalently bound FAD molecule. RoLAAO was crystallized by the vapour-diffusion method in two different space groups: $P2_12_12_1$ (unit-cell parameters a = 65.7, b = 109.7, c = 134.4 Å) and $C222_1$ (unit-cell parameters a = 68.3, b = 88.4, c = 186.6 Å). Both crystal forms diffracted X-rays to a resolution of at least 1.6 Å.

1. Introduction

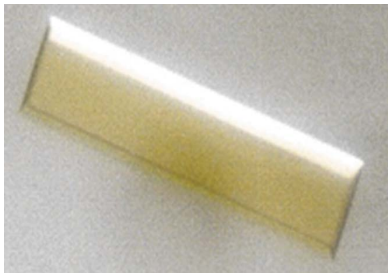
L-Amino-acid oxidases (EC 1.4.3.2; LAAOs) are dimeric flavoproteins containing non-covalently bound FAD as a cofactor. This enzyme is found in snake and insect venoms as well as in fungi, algae, cyanobacteria and soil bacteria. The first LAAOs to be described (Zeller & Maritz, 1944) were from snake venoms. These are also the best investigated representatives of this class of enzymes (Ponnudurai *et al.*, 1994; Sanchez & Magalhaes, 1991; Souza *et al.*, 1999; Tan & Saifuddin, 1991; Tan & Swaminathan, 1992; Torii *et al.*, 1994).

LAAOs are classified as N-terminal FAD-bound reductases. Classification of enzymes into this family is based on the presence of two highly conserved dinucleotide-binding motifs (Vallon, 2000). Other representatives of this family are cholesterol oxidase, *p*-hydroxybenzoate hydrolase, the sarcosine, polyamine and glucose oxidases, and phenyl hydroxylase.

L-Amino-acid oxidases catalyse the stereospecific oxidative deamination of an L-amino-acid substrate to the corresponding α-keto acid, with the production of ammonia and hydrogen peroxide (Fig. 1).

Most LAAOs accept a broad range of L-amino-acid substrates. In contrast, there are several LAAOs with a strict preference for specific substrates like L-glutamate oxidase from *Streptomyces* (Böhmer *et al.*, 1989; Kamei, Asano, Kondo *et al.*, 1983; Kamei, Asano, Suzuki *et al.*, 1983) or L-phenylalanine oxidase from *Pseudomonas* (Koyama, 1983) which are named after their favoured substrates.

The first LAAO three-dimensional structure was determined for the snake-venom LAAO from *Calloselasma rhodostoma* (Pawelek *et al.*, 2000). Recently, the structure of LAAO from *Agkistrodon halys*



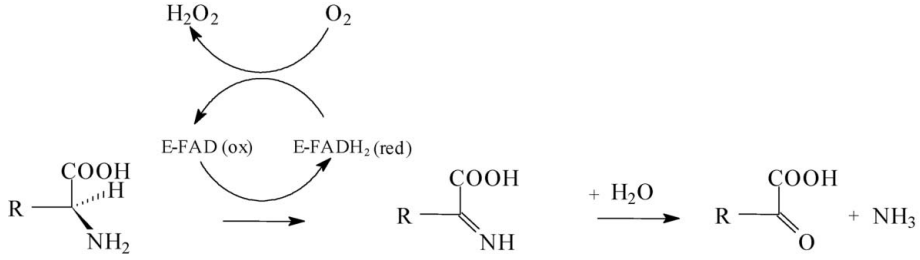


Figure 1
Schematic representation of the reaction catalysed by LAAO.

pallas, which shares 86% sequence identity to that from *C. rhodostoma*, was published (Zhang *et al.*, 2004). The LAAO from *Rhodococcus opacus*, which we examine in this study, shows a sequence identity of about 23–25% to the snake-venom enzymes.

Little is known about the function of LAAOs from prokaryotes and lower eukaryotes. LAAO from the fungus *Neurospora crassus* is induced by lack of readily metabolizable nitrogenous compounds in the presence of L-arginine and L-phenylalanine (DeBusk & Ogilvie, 1984). The LAAO from *Chlamydomonas reinhardtii* is induced if there is no primary nitrogen source available to produce ammonia (Munoz-Blanco *et al.*, 1990). Therefore, it is assumed that these inducible LAAOs play a role in the nitrogen supply to cells. It may be that in general these LAAOs are necessary in nitrogen and L-amino-acid metabolism.

In snake venoms, the enzyme is present at high concentrations and is postulated to be a toxin (Li *et al.*, 1994; Torii *et al.*, 1994). LAAOs from *Crotalus* can associate specifically with mammalian endothelial cells (Suhr & Kim, 1996, 1999). These LAAOs induce apoptosis in these cells, probably because of the high local concentration of hydrogen peroxide. It is reported that the toxicity of LAAO from snake venoms can be reduced by the addition of catalase (Takatsuka *et al.*, 2001; Torii *et al.*, 1994).

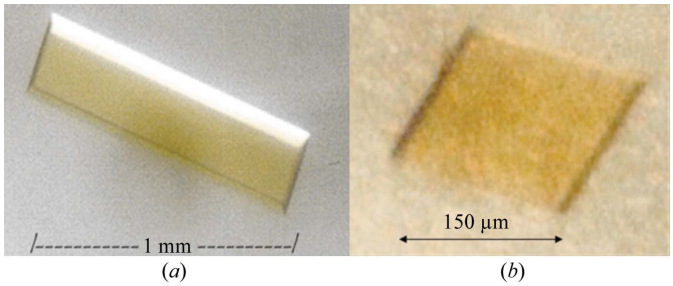
In this study, we describe the crystallization and preliminary X-ray analysis of the L-amino-acid oxidase from the soil bacteria *R. opacus* (RoLAAO). RoLAAO consists of 489 amino acids per monomer, with a calculated molecular weight of 54.2 kDa. Based on gel-filtration experiments, the enzyme was proposed to be a homodimer. The three-dimensional structure of LAAO from *R. opacus* could be useful in understanding the differing functionalities of LAAO in venoms and bacteria.

With structural knowledge of the corresponding DAAOs from maize and pig kidney being available (Mattevi *et al.*, 1996; Mizutani *et al.*, 2000; Pollegioni *et al.*, 2002; Umhau *et al.*, 2000), a structure of a bacterial LAAO could provide interesting information regarding the differences in mechanism as well as in substrate specificity of the enzymes.

2. Experimental

2.1. Protein expression and purification

Wild-type RoLAAO was overexpressed in *R. opacus* (strain DSM 43250) and purified as described previously (Geueke & Hummel, 2002). The protein solution was concentrated to 10–15 mg ml^{-1} in 50 m*M* glycine buffer pH 8.6 and stored at 277 K. The homogenity of the protein was checked by SDS–PAGE, native PAGE and ESI–MS.



(*a*) (*b*)

Figure 2
(*a*) Crystal of the primitive orthorhombic crystal form. (*b*) Crystal of the centred orthorhombic crystal form.

2.2. Crystallization experiments

All crystallization experiments were performed at 285 K using the sitting-drop variant of the vapour-diffusion method. Initial crystallization conditions were screened using the sparse-matrix approach (Jancarik & Kim, 1991). Crystal plates and clusters were obtained under several conditions. The most promising crystallization conditions were optimized by microseeding techniques. 4 µl droplets were typically used for crystallization experiments. The drops were prepared by mixing 2 µl protein solution with an equal volume of the reservoir solution and these drops were equilibrated against 1 ml reservoir solution.

2.3. Diffraction experiments

For X-ray diffraction experiments at cryogenic temperatures, the primitive orthorhombic crystals were transferred stepwise into a cryoprotectant buffer consisting of the reservoir solution containing up to 20%(*v*/*v*) MPD. Crystals grown with MPD could be mounted directly from the drop without any further addition of cryoprotectant. For derivatization, crystals of the primitive orthorhombic space group were exclusively used. Native crystals were soaked in crystallization buffer containing 10 m*M* $HgCl_2$ for 24 h and soaked for 30 min in crystallization buffer before cryosoaking in order to remove unspecifically bound heavy atoms. The crystals were mounted in nylon loops and flash-cooled in liquid nitrogen. The raw diffraction data were indexed, processed and scaled with *DENZO* and *SCALEPACK* from the *HKL* suite (Otwinowski, 1993).

3. Results and discussion

3.1. Crystallization and characterization of the $P2_12_12_1$ crystal form

The $P2_12_12_1$ crystal form of native LAAO was obtained by the sitting-drop method described above using Crystal Screen I from Hampton Research. The reservoir solution consists of 100 m*M* HEPES pH 7.5, 10%(*v*/*v*) 2-propanol and 20%(*w*/*w*) PEG 4000. Large plates and clusters were initially observed after about 4 d. Optimization of the initial crystallization condition leads to a reservoir solution consisting of 100 m*M* HEPES pH 7.8, 10%(*v*/*v*) 2-propanol and 10%(*w*/*w*) PEG 4000. Microseeding was crucial in order to obtain well shaped $P2_12_12_1$ crystals. As the quality and the size of the crystals depended on the quality of the seed crystals, several cycles of microseeding were performed. In each cycle, the seeds were transferred directly into the optimized reservoir solution consisting of 10%(*w*/*w*) PEG 4000, 10%(*v*/*v*) 2-propanol and 100 m*M* HEPES pH 7.8. After 3 d equilibration of the drops, 0.5 µl seeding solution was added. Orthorhombic crystals appeared within hours and achieved dimensions of 200 × 200 × 100 µm (Fig. 2*a*).

A native 1.43 Å data set was collected at the X11 beamline at EMBL Outstation (Hamburg) using X-ray synchrotron radiation and a MAR CCD 165 mm detector (Table 1). The diffraction pattern indicated a primitive orthorhombic unit cell with unit-cell parameters $a = 65.7$, $b = 109.7$, $c = 134.4$ Å. Assuming the specific ratio of volume to protein in the crystal (V_M) to be within the normal range of values observed for soluble protein crystals (Matthews, 1968), the asymmetric unit contains a dimer (108.4 kDa; $V_M = 2.4$ Å^3 Da^{-1}).

3.2. Crystallization of the $C222_1$ crystal form

A second crystal form was observed during the refinement of the $P2_12_12_1$ crystals by exchanging the volatile 2-propanol for MPD. These crystals could be obtained either from an optimized reservoir solution containing 100 m*M* HEPES pH 7.8 and 35%(*v*/*v*) MPD by

Table 1
Statistics of data sets.

Values in parentheses are for the outermost resolution shell.

Space group	$P2_12_12_1$	$C222_1$
Unit-cell parameters (Å)		
a	65.7	68.3
b	109.7	88.4
c	134.4	186.6
Resolution range (Å)	25–1.4 (1.45–1.4)	50–1.64 (1.7–1.64)
Completeness (%)	99.7 (99.4)	96.4 (94.7)
$I/\sigma(I)$	43.0 (7.4)	21.2 (9.0)
R_{sym} (%)	6 (38.5)	4.2 (10.8)
No. of reflections	2398984	150035
No. of unique reflections	189334	67319
Multiplicity	34.4 (3.9)	2.4 (2.3)

cross-seeding with crushed and diluted $P2_12_12_1$ crystals or without seeding from a reservoir solution containing 42%(*v*/*v*) MPD. The crystals grew to dimensions of 200 × 300 × 100 µm within three months (Fig. 2*b*). A data set was collected to 1.64 Å resolution at the X13 beamline at the EMBL Outstation (Hamburg) using X-ray synchrotron radiation and a MAR CCD 165 mm detector. Data could be indexed in the centric orthorhombic space group $C222_1$, with unit-cell parameters *a* = 68.3, *b* = 88.4, *c* = 186.6 Å. The calculated V_M value for one monomer in the asymmetric unit is 2.6 Å^3 Da^{-1}, meaning that the RoLAAO dimer should occupy a special position on a crystallographic twofold axis.

3.3. Experimental phasing

A SAD data set of a mercury derivative was obtained at 1.008 Å on beamline X31 at EMBL Outstation, Hamburg with a MAR 345 image-plate detector (data not shown). Four mercury sites could be located using the automated protocol of *autoSHARP*/*SHARP* (Bricogne *et al.*, 2003) and initial phasing resulted in an interpretable electron-density map. For all data sets, the data-collection strategies were calculated using the program *BEST* (Popov & Bourenkov, 2003).

This study was supported by the Deutsche Forschungsgemeinschaft (DFG, grant SCHO 245-12-1). We would like to thank A. Popov and M. Groves (EMBL Outstation Hamburg) for their excellent support during data collection.

References

Böhmer, A., Müller, A., Passarge, M., Liebs, P., Honeck, H. & Müller, H. G. (1989). *Eur. J. Biochem.* **182**, 327–332.
Bricogne, G., Vonrhein, C., Flensburg, C., Schiltz, M. & Paciorek, W. (2003). *Acta Cryst.* D**59**, 2023–2030.
DeBusk, R. M. & Ogilvie, S. (1984). *J. Bacteriol.* **159**, 583–589.
Geueke, B. & Hummel, W. (2002). *Enyzme Microbiol. Technol.* **31**, 77–87.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
Kamei, T., Asano, K., Kondo, H., Matsukaki, M. & Nakamura, S. (1983). *Chem. Pharm. Bull.* **31**, 3609–3616.
Kamei, T., Asano, K., Suzuki, H., Matsukaki, M. & Nakamura, S. (1983). *Chem. Pharm. Bull.* **31**, 1307–1314.
Koyama, H. (1983). *J. Biochem. (Tokyo)*, **93**, 1313–1319.
Li, Z. Y., Yu, T. F. & Lian, E. C. Y. (1994). *Toxicon*, **32**, 1349–1358.
Mattevi, A., Vanoni, M. A., Todone, F., Rizzi, M., Teplyakov, A., Coda, A., Bolognesi, M. & Curti, B. (1996). *Proc. Natl Acad. Sci. USA*, **93**, 7496–7501.
Matthews, B. (1968). *J. Mol. Biol.* **33**, 491–497.
Mizutani, H., Miyahara, I., Hirotsu, K., Nishina, Y., Shiga, K., Setoyama, C. & Miura, R. (2000). *J. Biochem (Tokyo)*, **128**, 73–81.
Munoz-Blanco, J., Hidalgo-Martinez, J. & Cardenas, J. (1990). *Planta*, **182**, 194–198.
Otwinowski, Z. (1993). *Proceedings of the CCP4 Study Weekend. Data Collection and Processing*, edited by L. Sawyer, N. Isaacs & S. Bailey, pp. 56–62. Warrington: Daresbury Laboratory.
Pawelek, P. D., Cheah, J., Coulombe, R., Macheroux, P., Ghisla, S. & Vrielink, A. (2000). *EMBO J.* **19**, 4204–4215.
Pollegioni, L., Diederichs, K., Molla, G., Umhau, S., Welte, W., Ghisla, S. & Pilone, M. S. (2002). *J. Mol. Biol.* **324**, 535–546.
Ponnudurai, G., Chung, M. C. & Tan, N. H. (1994). *Arch. Biochem. Biophys.* **313**, 373–378.
Popov, A. N. & Bourenkov, G. P. (2003). *Acta Cryst.* D**59**, 1145–1153.
Sanchez, E. & Magalhaes, A. (1991). *Braz. J. Med. Biol. Res.* **24**, 249–260.
Souza, D. H., Eugenio, L. M., Fletcher, J. E., Jiang, M. S., Garratt, R. C., Oliva, G. & Selistre-de-Araujo, H. S. (1999). *Arch. Biochem. Biophys.* **368**, 285–290.
Suhr, S. M. & Kim, D. S. (1996). *Biochem. Biophys. Res. Commun.* **224**, 134–139.
Suhr, S. M. & Kim, D. S. (1999). *J. Biochem.* **125**, 305–309.
Takatsuka, H., Sakurai, Y., Yoshioka, A., Kokubo, T., Usami, Y., Suzuki, M., Matsui, T., Titani, K., Yagi, H., Matsumoto, M. & Fujimura, Y. (2001). *Biochem. Biophys. Acta*, **1544**, 267–277.
Tan, N. & Saifuddin, N. (1991). *Int. J. Biochem.* **23**, 323–327.
Tan, N. & Swaminathan, S. (1992). *Int. J. Biochem.* **24**, 967–973.
Torii, S., Naito, M. & Tsuruo, T. (1994). *J. Biol. Chem.* **272**, 9539–9542.
Umhau, S., Pollegioni, L., Molla, G., Diederichs, K., Welte, W., Pilone, M. S. & Ghisla, S. (2000). *Proc. Natl Acad. Sci. USA*, **97**, 12463–12468.
Vallon, O. (2000). *Proteins*, **38**, 95–114.
Zeller, A. & Maritz, A. (1944). *Helv. Chim. Acta*, **27**, 1888–1902.
Zhang, H., Teng, M., Niu, L., Wang, Y., Wang, Y., Huang, Q., Hao, Q., Dong, Y. & Liu, P. (2004). *Acta Cryst.* D**60**, 974–977.