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Published in:
Acta crystallographica. Section D: Biological crystallography

DOI:
10.1107/S0907444902015743

Publication date:
2002

Document Version
Publisher's PDF, also known as Version of record

Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Overexpression, purification, crystallization and preliminary X-ray crystallographic analysis of *Pseudomonas aeruginosa* L-arginine deiminase

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Received 10 June 2002
Accepted 2 September 2002

Pseudomonas aeruginosa L-arginine deiminase, an enzyme catalyzing the irreversible catabolism of arginine to citrulline, has been produced in selenomethionyl form. The protein was purified and crystallized by the sitting-drop vapour-diffusion method using a precipitant solution consisting of 55% MPD, 100 m*M* cacodylate pH 6.5, 20 m*M* $MgCl_2$. Crystals display tetragonal symmetry ($P4_12_12$ or $P4_32_12$), with unit-cell parameters $a = b = 106.0$, $c = 300.2$ Å, and diffract to 2.7 Å resolution. A complete MAD data set was collected to 3.2 Å resolution on beamline BM30 at ESRF.

1. Introduction

Pseudomonas aeruginosa (PAO), a Gram-negative bacterium, is an opportunistic human pathogen that causes a wide variety of infections, particularly in victims of severe burns and in immunosuppressed patients. This makes *P. aeruginosa* one of the most prevalent pathogens in hospital-acquired infection. The potential pathogenicity of PAO is greatly enhanced by a remarkable nutritional versatility that enables this organism to survive in diverse and harsh environments.

The significance of arginine catabolism in this organism is reflected by the unusual presence of four pathways for its utilization. *P. aeruginosa* can utilize arginine aerobic conditions as a sole source of carbon, energy and nitrogen *via* the arginine succinyltransferase pathway (Cunin *et al.*, 1986). PAO also utilizes arginine as an energy source under anaerobic conditions *via* the L-arginine deiminase (ADI; EC 3.5.3.6) pathway.

In *P. aeruginosa* the genetics of the arginine deiminase pathway have been well studied. The genes encoding the enzyme of the ADI pathway are organized in an *arcDABC* operon (Rella *et al.*, 1985; Vander Wauven *et al.*, 1984). Expression of this operon is induced under conditions of oxygen limitation (Mercenier *et al.*, 1980).

The *arcA* gene encodes the arginine deiminase (EC 3.5.3.6) that catalyzes the deimination of the guanidino group of L-arginine to produce citrulline and ammonia. The catabolic ornithine carbamoyltransferase (EC 2.1.3.3), encoded by the *arcB* gene, cleaves citrulline to ornithine and carbamoyl phosphate. The *arcC* gene encodes the carbamate kinase (EC 2.7.2.2) that catalyzes the formation of ATP from carbamoyl phosphate and ADP. The fourth gene, *arcD*, encodes a membrane-bound protein that is necessary for the uptake of arginine and the excretion of ornithine. This transport process is driven by a concentration gradient (Lüthi *et al.*, 1990; Verhoogt *et al.*, 1992).

The arginine deiminase pathway, normally confined to the prokaryotic kingdom, has also been discovered in the primitive eukaryotic organism *Giardia intestinalis* and is an important fuel in its overall energy metabolism (Edwards *et al.*, 1992). ADI from *G. intestinalis* (580 amino acids) shares about 60% sequence homology with the PAO enzyme (417 amino acids), but contains an extra segment (about 120 residues) on its C-terminus.

Protozoa of the genus *Giardia* are ubiquitous parasites that colonize the mucosa of the gastrointestinal tracts of human and animals (Adam, 1991). A number of different classes of drugs have been successful in therapy for giardiasis worldwide (Upcroft & Upcroft, 2001). Resistance to metronidazole in *G. intestinalis* has been documented (Adagu *et al.*, 2002). Should resistance to metronidazole or its analogue become widespread, new active compounds will be needed.

The search for alternative antigiardials, which relies on the intimate knowledge of *Giardia* biochemistry, metabolism and molecular biology, is essential. Consequently, arginine deiminase could be a potential therapeutic target for the treatment of *Giardia* infection.

In both *P. aeruginosa* and *Giardia*, L-arginine deiminase appears to be a key enzyme and a drug target for potential antibacterial or antigiardials design. As the first step towards structure elucidation of this enzyme, we report here the overexpression, crystallization and preliminary X-ray diffraction studies of *P. aeruginosa* ADI.

2. Material and methods

2.1. Cloning and overexpression

The vector pET30.b (Novagen) was used for ADI expression. The *arcA* gene encoding the *P. aeruginosa* arginine deiminase was amplified by PCR using pME183 as template (Lüthi *et al.*, 1986). The forward (5′-GGCCTCGAGTCAGTAGTCGATCG-GGTCGCGG-3′) and reverse (5′-GGC-ATATGAGCACGGAAAAAACCAAAC-TTGGCG-3′) oligonucleotide primers were designed using the published sequence (Baur *et al.*, 1989).

The PCR product was initially cloned into pCR2.1 (Invitrogen); after sequence confirmation, the gene was excized and subcloned into pET30.b as an *Nde*I/*Eco*RI fragment. The plasmid encoding arginine deiminase was transformed into *Escherichia coli* strain BL21(DE3)pLysS for protein expression.

To help structure solution, selenomethionyl (SeMet) ADI was produced using the method described by Doublié (1997). Expression of the recombinant enzyme was induced overnight at 288 K using 1 m*M* isopropyl-thio-β-D-galactoside.

Cells from 4 l of culture were resuspended in 20 m*M* Tris–HCl pH 7.4, 10% glycerol, 0.2 m*M* EDTA, 3 m*M* β-mercaptoethanol. Cell disruption was achieved by sonication. Debris was removed by centrifugation at 12 000*g* for 20 min.

2.2. Purification

Recombinant SeMet-containing *P. aeruginosa* arginine deiminase was purified by a three-step column chromatography procedure: (i) a DEAE-Sepharose column equilibrated with 10 m*M* Tris–HCl, 10% glycerol, 0.2 m*M* EDTA, 3 m*M* β-mercaptoethanol was eluted using a linear gradient of 0–400 m*M* $MgCl_2$; (ii) an arginine Sepharose column pre-equilibrated with the same sample was eluted with a linear gradient of arginine (0–200 m*M*); (iii) the most active fractions (Archibald, 1944) were applied to a column of Resource Q. Arginine deiminase was eluted with a 0–150 m*M* gradient of $MgCl_2$.

Fractions were analyzed by SDS–PAGE (Fig. 1) and *in vitro* enzyme assay (Archibald, 1944). The yield of the SeMet enzyme is 30 mg per litre of culture. Mass spectrometry (data not shown) confirmed the incorporation of selenium into the protein.

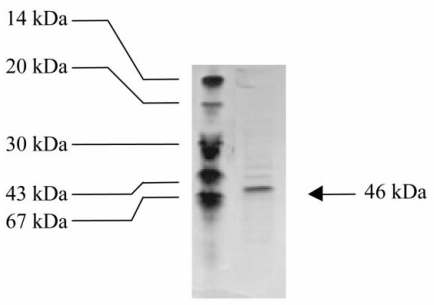

Figure 1
Coomassie-stained SDS–PAGE (8–25%) gel on a purified fraction of *P. aeruginosa* ADI showing a single band at the expected MW of about 46 kDa.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

	Inflection	Peak	Remote	High-resolution pass
Wavelength (Å)	0.97905	0.97888	0.97243	0.97888
Resolution (Å)	99–3.24 (3.32–3.24)	99–3.24 (3.32–3.24)	99–3.22 (3.30–3.22)	99–2.70 (2.83–2.70)
No. measured reflections	277287	280949	285130	259812
No. unique reflections	59392	59374	61846	94298
Completeness (%)	99.2 (97.8)	99.4 (98.2)	99.2 (97.2)	91.1 (69.0)
R_{merge} (%)	9.9 (29.4)	10.0 (35.5)	9.7 (28.1)	8.0 (31.3)

2.3. Crystallization of selenomethionyl *P. aeruginosa* ADI

The purified enzyme samples were concentrated to about 10 mg ml^{-1} by ultrafiltration (YM30, Amicon) and the protein concentration was estimated by UV absorption based on a calculated absorption molar coefficient of 54 270 M^{-1} cm^{-1} at 280 nm (Kalckar & Shafran, 1947).

Crystallization attempts were performed by the sitting-drop vapour-diffusion method using 24-well tissue-culture VDX plates (Hampton Research) at 293 K. Initial searches for crystallization conditions were performed using the standard sparse-matrix crystal screens (Jancarik & Kim, 1991) from Hampton Research (Crystal Screen I and Crystal Screen II) and further refined.

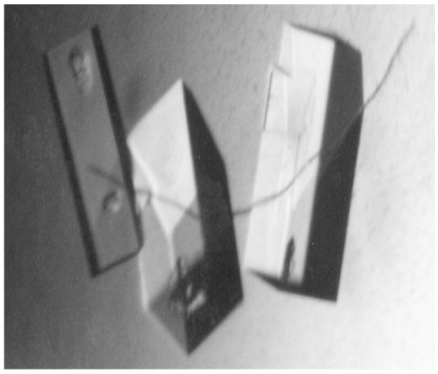

Figure 2
Typical plate crystals of PAO selenomethionyl arginine deiminase obtained by equilibration against a reservoir containing 55% MPD, 100 m*M* cacodylate pH 6.5, 20 m*M* $MgCl_2$. The maximum size of the crystals is about 0.400 mm.

Crystals of *P. aeruginosa* ADI emerged from several conditions. These conditions were refined and the best crystals were obtained with 100 m*M* sodium cacodylate buffer pH 6.5, 55% MPD and 20 m*M* $MgCl_2$. In each trial, a sitting drop of 5 µl of protein solution (10 mg ml^{-1} in 50 m*M* Tris–HCl buffer pH 7.4, 10% glycerol, 0.2 m*M* EDTA, 3 m*M* β-mercaptoethanol) mixed with 5 µl of well solution (100 m*M* sodium cacodylate buffer pH 6.5, 55% MPD) was equilibrated against a reservoir containing 600 µl of well solution. Typical crystals grew as regular blocks of dimensions 0.20 × 0.20 × 0.40 mm in about 3 d (Fig. 2).

3. Data collection and analysis

Preliminary diffraction data were collected on a MAR345 imaging-plate system from MAR Research equipped with Osmic optics and running on an FR591 rotating-anode generator (Cu $K\alpha$).

The crystals display tetragonal symmetry ($P4_12_12$ or $P4_32_12$), with unit-cell parameters $a = b = 106.0$, $c = 300.2$ Å. The large c cell parameter combined with the large mosaicity of the crystals caused the diffraction spots to overlap when the detector was too close to the crystal, limiting resolution.

Crystals were flash-frozen and diffraction data were collected on a MAR CCD detector on beamline BM30 at ESRF (Grenoble). Diffraction data were recorded using the multiwavelength anomalous diffraction (MAD) method in order to obtain initial phase information for structural determination. Prior to collection of the diffraction data, the X-ray fluorescence spectrum of the ADI crystal was measured in order to determine the absorption edge of selenium. Three data sets for MAD calculation were collected with wavelengths of 0.9788 (peak), 0.9790 (inflection) and 0.9724 Å (remote) using a single crystal. The distance between the crystal and the detector was set to 200 mm as a compromise between resolution and spot overlap. Indeed, the combination of the long c cell

parameter and high mosaicity limits the distance between the crystal and the detector and hence restricts the resolution of the data set. Under these conditions, the crystal diffracted to only about 3.20 Å. Data were processed with the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997) and the results are summarized in Table 1. The overall completeness at the three wavelengths is about 99.2% in the resolution range 99.0–3.24 Å, with overall $R_{\rm sym}$ values below 10.0%.

No serious radiation damage to the crystal was detected during the first data collection at the three different wavelengths and therefore a higher resolution pass was recorded on the same crystal by moving the ω angle of the detector to 10°, leading to a set with slightly higher resolution. For this second data set, the statistics showed an overall completeness of only 91.1% in the resolution range 99.0–2.70 Å, with an overall $R_{\rm sym}$ of 8.0% and an $R_{\rm sym}$ of 31.3% for the last shell (2.83–2.70 Å).

4. Structure analysis

Biochemical studies suggest that the active arginine deiminase is a tetrameric protein with a molecular weight of about 46 kDa per monomer (Baur *et al.*, 1989). Based on the sequence of the enzyme, 11 (Se)Met residues are expected for each protein subunit.

According to the cell size and symmetry, the solvent content is calculated to be 51.3%, assuming four molecules in the asymmetric unit and a density of 1.30 g ml^{-1}. The Matthews coefficient (Matthews, 1968) is 2.622 Å^3 Da^{-1} based on a molecular weight of 46 400 Da per subunit for ADI.

We thank the members of the FIP BM30 beamline, ESRF for advice on MAD data collection and Rudy Watthiez for mass-spectrometry analysis of the selenomethionyl ADI samples. We acknowledge support from the Fonds National de la Recherche Scientifique (IISN grant No. 4.4.505.00.F). This work was also supported in part by an 'Action de Recherche Concertée' (ARC) financed by the French Community of Belgium and from the Belgian Fund for Joint Basic research.

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