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## Crystallization, diffraction data collection and preliminary crystallographic analysis of hexagonal crystals of *Pseudomonas aeruginosa* amidase

The aliphatic amidase (acylamide amidohydrolase; EC 3.5.1.4) from *Pseudomonas aeruginosa* is a hexameric enzyme composed of six identical subunits with a molecular weight of ~38 kDa. Since microbial amidases are very important enzymes in industrial biocatalysis, the structural characterization of this enzyme will help in the design of novel catalytic activities of commercial interest. The present study reports the successful crystallization of the wild-type amidase from *P. aeruginosa*. Native crystals were obtained and a complete data set was collected at 1.4 Å resolution, although the crystals showed diffraction to 1.25 Å resolution. The crystals were found to belong to space group  $P6_322$ , with unit-cell parameters  $a = b = 102.60$ ,  $c = 151.71$  Å, and contain one molecule in the asymmetric unit.

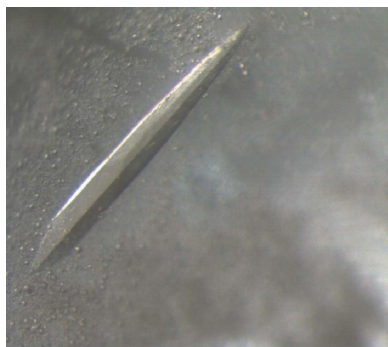
### 1. Introduction

Powerful new advances in protein technology and commercial enzyme applications are continually being proposed (Kirk *et al.*, 2002; Lorenz & Eck, 2005). Efforts to understand the relationship between protein structure and function (Banerjee *et al.*, 2002) are essential for the manipulation and creation of novel catalytic activities that could be used as commercial biocatalysts (Li *et al.*, 2005).

The aliphatic amidase (acylamide amidohydrolase; EC 3.5.1.4) encoded by the *amiE* gene from *Pseudomonas aeruginosa* is an inducible enzyme that catalyses the hydrolysis of small aliphatic amides (principally C<sub>1</sub>–C<sub>4</sub>; Brown *et al.*, 1969; Brown & Clarke, 1972; Brown & Tata, 1987; Pacheco *et al.*, 2005). Although the detailed catalytic mechanism is still not totally understood (Findlater & Orsi, 1973; Woods *et al.*, 1979; Novo *et al.*, 2002), this enzyme has attracted great attention because of the variations in its substrate and inhibitor specificities that can be generated by single-point mutations (Tata *et al.*, 1994; Karmali *et al.*, 2000, 2001; Gregoriou & Brown, 1979, 1980). Microbial amidases with altered substrate specificities can be used in several industrial applications such as the detoxification of industrial effluents containing toxic amides and the production of hydroxamic acids and other organic acids (Fournand *et al.*, 1998). Hydroxamic acids are known for their chelating properties (Vanjari & Pande, 2003) and some of them have been described as potent inhibitors of metalloproteases and have been investigated as anti-human immunodeficiency virus agents or antimalarial agents (Gao *et al.*, 1995; Tsafack *et al.*, 1996).

As the substrate specificity of this enzyme can be changed by single-point mutations, we began the structural characterization of amidase from *P. aeruginosa*. It would be of great interest to understand the structural changes responsible for the differences in substrate and inhibitor specificities in order to help in the design of new *in vitro* mutations to create amidases with novel specificities of commercial interest.

Recently, a preliminary structure determination of the amidase from *Geobacillus pallidus* RAPc8 was described (Agarkar *et al.*,



**Table 1**

Statistics of diffraction data collection.

Values in parentheses are for the outer resolution shell. Two resolution ranges are given for the synchrotron data, as the data for the outer resolution shell were incompletely collected.

X-ray source	ITQB, Bruker– Nonius FR570	ESRF, ID29	
Temperature (K)	110.0	100.0	
Wavelength (Å)	1.5418	0.9756	
Space group	<i>P</i> <sub>6</sub> <sub>3</sub> <sub>2</sub> <sub>2</sub>	<i>P</i> <sub>6</sub> <sub>3</sub> <sub>2</sub> <sub>2</sub>	
Unit-cell parameters (Å)	<i>a</i> = <i>b</i> = 101.69, <i>c</i> = 151.43	<i>a</i> = <i>b</i> = 102.71, <i>c</i> = 151.32	
Mosaicity (°)	0.76–0.88	0.088–0.176	
Wilson <i>B</i> factor (Å <sup>2</sup> )	19.4	8.5	
Resolution range (Å)	26.02–1.78 (1.84–1.78)	42.67–1.40 (1.43–1.40)	42.67–1.25 (1.28–1.25)
No. of reflections	312413	1382592	1545027
No. of unique reflections	44460	91754	115216
Redundancy	7.0	15.1	13.4
No. of rejected outliers (%)	180 (0.06)	1888 (0.14)	2035 (0.13)
Completeness (%)	98.8 (95.0)	99.0 (88.9)	89.0 (51.5)
<i>I</i> /σ( <i>I</i> )	27.0 (5.8)	28.5 (7.3)	24.9 (3.0)
<i>R</i> <sub>p.i.m.</sub> † (%)	2.6 (13.3)	2.1 (10.6)	2.2 (26.0)
<i>R</i> <sub>r.i.m.</sub> ‡ (%)	7.1 (34.7)	8.6 (33.5)	9.0 (56.8)
<i>R</i> <sub>sym</sub> § (%)	6.6 (32.0)	8.4 (31.5)	8.7 (49.7)

†  $R_{p.i.m.} = \sum_h [1/(N-1)]^{1/2} \sum_i |I_i(h) - \langle I(h) \rangle| / \sum_h \sum_i I_i(h)$ , where *I* is each observed intensity and  $\langle I \rangle$  is the average intensity of multiple observations from symmetry-related reflections. Calculated using the program *RMERGE* (Weiss, 2001), it is an indicator of the precision of the final merged and averaged data set. ‡  $R_{r.i.m.} = R_{meas} = \sum_h [N/(N-1)]^{1/2} \sum_i |I_i(h) - \langle I(h) \rangle| / \sum_h \sum_i I_i(h)$ , where *I* is each observed intensity and  $\langle I \rangle$  is the average intensity of multiple observations of symmetry-related reflections. Calculated using the program *RMERGE* (Weiss, 2001), it represents an indicator of the average spread of the individual measurements. §  $R_{sym} = \sum_h \sum_i |I_i(h) - \langle I(h) \rangle| / \sum_h \sum_i I_i(h)$ , where *I* is the observed intensity and  $\langle I \rangle$  is the average intensity of multiple observations of symmetry-related reflections. *R*<sub>sym</sub> was calculated using *SCALEPACK* (Otwinowski & Minor, 1997).

2006). Despite an earlier description of crystals from *P. aeruginosa* amidase belonging to the rhombohedral space group *R*3 or *R*32 and diffracting to 3 Å resolution (Farnaud *et al.*, 1999), no experimental structural characterization of the *P. aeruginosa* amidase has subsequently been published. In this paper, we report the crystallization of a hexagonal form of *P. aeruginosa* amidase crystals, their diffraction to quasi-atomic resolution and their crystallographic characterization.

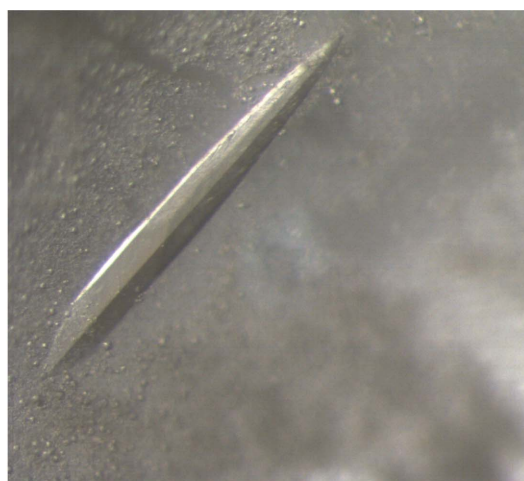
## 2. Materials and methods

### 2.1. Protein expression and purification

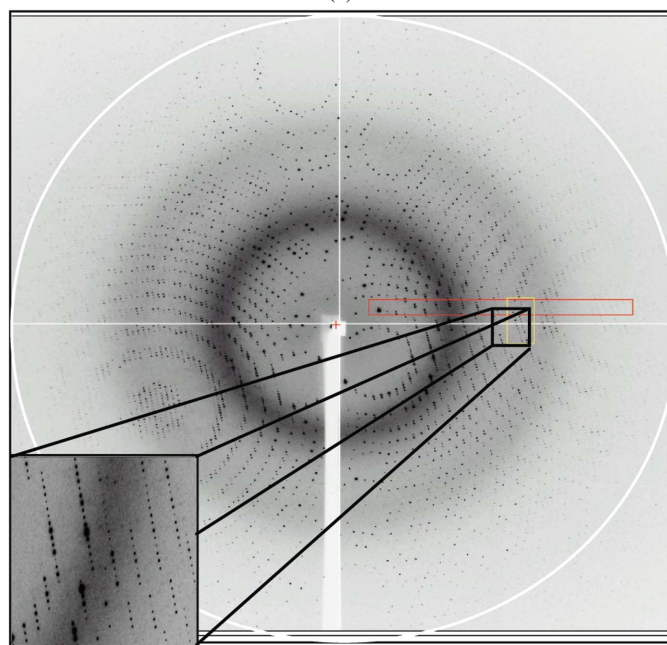
A constitutive mutant strain L10 of *P. aeruginosa* was grown as reported previously (Domingos *et al.*, 1989). The wild-type amidase from *P. aeruginosa* was extracted from cells and purified by a two-step procedure involving affinity and gel-filtration chromatography as described previously (Domingos *et al.*, 1989; Karmali *et al.*, 2001; Martins *et al.*, 2005) with some modifications. The elution of amidase activity from the affinity column (epoxy-activated Sepharose 6B acetamide) was carried out with a linear gradient of acetamide and hydroxylamine (0–40 mM) in 20 mM Tris–HCl buffer pH 7.2 containing 1 mM β-mercaptoethanol, 1 mM EDTA, 10% (v/v) glycerol and 1 mM benzamidine (TMEGB). The fractions containing amidase activity were pooled, concentrated using a P30 membrane (Amicon) and applied onto a column (2 × 100 cm) packed with Sephacryl S300HR (Amersham Pharmacia) which had been previously equilibrated with TMEGB. Amidase activity was eluted with the same buffer system and the peak fractions containing amidase activity were analysed by native PAGE and SDS–PAGE, dialyzed against 50 mM Tris–HCl pH 7.2 containing 5 mM DTT and 1 mM EDTA, concentrated to 25 mg ml<sup>−1</sup> and stored at 253 K until use.

### 2.2. Protein crystallization

In addition to crystals obtained under conditions similar to those described for *P. aeruginosa* wild-type amidase (Farnaud *et al.*, 1999), new crystallization trials using screening kits (Nextal Biotech) with a Cartesian Dispensing System (Genomic Solutions) delivered a new crystal form with enhanced diffraction resolution. This was further optimized by the vapour-diffusion method using sitting drops in VDX plates (Hampton Research) by mixing 5 μl of the purified enzyme at 25 mg ml<sup>−1</sup> (see §2.1) with 2 μl well solution, 15% (v/v) PEG 4K and 5% (v/v) saturated solution of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> pH 6.8. Drops were allowed to equilibrate against 500 μl reservoir solution at 305 K. Crystals appeared within a few hours and reached their maximum dimensions within 24–30 h. Appropriate cryoprotection conditions were established by a fast soak (up to 30 s) of each crystal in crystallization solution complemented with 25% (v/v) glycerol prior to flash-cooling in liquid nitrogen.



(a)



(b)

**Figure 1**  
(a) Elongated polyhedral crystal of *P. aeruginosa* amidase, reaching dimensions of 0.7 × 0.1 × 0.1 mm. (b) X-ray diffraction pattern from an amidase crystal using synchrotron radiation at beamline ID29, ESRF, Grenoble. The white circumference marks 1.5 Å resolution and the detector corner corresponds to 1.25 Å resolution.

## 2.3. Diffraction data collection and crystal characterization

The new hexagonal crystals reach maximum dimensions of  $0.7 \times 0.1 \times 0.1$  mm (Fig. 1a) and appear as yellowish elongated polyhedra. Their light colouring is in accordance with that observed for a concentrated solution of the purified amidase, which was homogeneous both on SDS-PAGE and native PAGE. Up to 24 h after setting up the crystallization drops, crystals were fished out with a loop, cryoprotected and stabilized under liquid nitrogen. Diffraction data sets were collected from native crystals in-house and at beamline ID29, ESRF, Grenoble, France. Diffraction intensities (see Table 1 for data-collection details and statistics) were examined with *XdisplayF*, processed with *DENZO* and scaled and merged with *SCALEPACK* from the *HKL* suite (Otwinowski & Minor, 1997).

## 3. Results and conclusions

A new hexagonal form of *P. aeruginosa* amidase crystals was obtained within 1 d of crystallization setup. The crystals diffracted to better than  $1.78 \text{ \AA}$  when using an X-ray home source, but achieved  $1.25 \text{ \AA}$  resolution when using synchrotron radiation. The low reproducibility of the rapid cryoprotection protocol may explain the 1% discrepancy in unit-cell parameters between the measured data sets. The light yellowish colouring of the crystals is in accordance with that observed for a concentrated solution of the purified amidase, which was homogeneous both on SDS-PAGE and native PAGE. Unused crystals remaining in the unperturbed crystallization drops turned to gel-like soft solids within 4–7 d and eventually totally lost their diffraction capability. These high-resolution *P. aeruginosa* crystals belong to space group  $P6_322$ , with unit-cell parameters  $a = b = 102.71$ ,  $c = 151.32 \text{ \AA}$ , which corresponds to a calculated solvent content of 52% (Matthews, 1968) assuming the presence of one amidase molecule per asymmetric unit. Therefore, it is possible that the biologically active *P. aeruginosa* homohexameric amidase (Farnaud *et al.*, 1999) is made up of monomers in a spatial arrangement similar to the quaternary structure described for the *G. pallidus* RAPc8 amidase (Agarkar *et al.*, 2006), a trimer of dimers exhibiting  $D3$  point-group symmetry.

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