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Purification, crystallization and preliminary X-ray crystal structure analysis of copper amine oxidase from *Arthrobacter globoformis*

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Abstract

β-Phenylethylamine oxidase from the Gram-positive bacterium *Arthrobacter globoformis* has been crystallized as three crystal forms. Two belong to space group $C2$ and one to space group $P2_12_12_1$, respectively. The unit-cell volumes are consistent with one subunit of 70 644 Da per asymmetric unit for the two monoclinic forms, and with two subunits per asymmetric unit for the orthorhombic crystals. Three-dimensional intensity data have been recorded to 2.8 Å resolution for one of the monoclinic crystal forms and to 3 Å resolution for the orthorhombic crystal form.

1. Introduction

Copper amine oxidases (AO's) catalyze the oxidative deamination of primary amines to the corresponding aldehyde,

$$R\mathrm{CH_2NH_2} + \mathrm{O_2} + \mathrm{H_2O} \xrightarrow{\mathrm{AO}} R\mathrm{CHO} + \mathrm{NH_3} + \mathrm{H_2O_2}.$$

The redox reaction is facilitated by a novel covalently bound organic cofactor, 6-hydroxydopa quinone (also known as topa quinone) derived from the post-translational modification of a tyrosine residue. AO's are distributed over a wide range of organisms and tissues (McIntire & Hartman, 1993; McIntire, 1994). In microorganisms, amine metabolism catalyzed by monoamine oxidases (MAO's) provides carbon and nitrogen for growth. In higher organisms, diamine oxidases (DAO's) participate in the catabolism of di- and polyamines which are important agents in many fundamental cellular processes, *e.g.* tissue growth and tissue differentiation, and tumour growth (for a recent review, see McIntire & Hartman, 1993). The rational design of specific inhibitors for AO's could lead to potent drugs against cancer and developmental diseases.

Copper amine oxidases are homodimers of 60–105 kDa subunits. The amine oxidases from different sources, including human kidney amiloride binding protein, have pair-wise sequence identity of at least 21% (Tanizawa, Matsuzaki, Shimizu, Yorifuji & Fukui, 1994; Novotny, Chassander, Baker, Lazdunski & Barbry, 1994), as well as closely similar physicochemical and mechanistic properties. The spectroscopic properties of the protein are consistent with 'type-2' or 'non-blue' copper sites. Despite the availability of a large body of spectroscopic and chemical data, the exact role of the copper remains unclear. Both a catalytic (Dooley *et al.*, 1991) and a structural role (Agostinelli, Morpurgo, Wang, Giartosio & Mondovi, 1994) have been suggested. The structure analyses of the native enzyme and substrate analogue/inhibitor complexes should help to resolve these questions, and elucidate the reaction pathway. A further uncertainty arises from conflicting evidence whether or not the copper sites in the two subunits are identical (Barker *et al.*, 1979; Suzuki, Sakurai, Nakahara, Manabe & Okuyama, 1986; Morpurgo, Agostinelli, Befani & Mondovi, 1987; Collison *et al.*, 1989; Agostinelli *et al.*, 1994). The present work resolves this question, at least for the case of *A. globoformis* amine oxidase (see *X-ray analysis*, below).

The crystallizations of DAO from pea seedlings (Vignevich *et al.*, 1993) and of MAO from *Escherichia coli* (Roh *et al.*, 1994) have been reported previously. We here report the crystallization and preliminary X-ray analysis of β-phenylethylamine oxidase (PEAO) from the Gram-positive bacterium *A. globoformis*.

2. Purification and crystallization

A. globoformis was grown in media containing β-phenylethylamine as described by Shimizu, Ichise & Yorifuzi (1990). The frozen cell paste was suspended in 16 m*M* potassium phosphate, pH 7.0, containing leupeptin, aprotinin, pepstatin and phenylmethylsulfonyl-fluoride as protease inhibitors, and lysozyme. The cells were ruptured by a combination of sonication and French pressure cell treatment. After centrifugation and dialysis, the sample was chromatographed on a DEAE–cellulose column with a 0–500 m*M* KCl gradient. The major PEAO fraction was subjected to ammonium sulfate (AS) fractionation. Most of the activity precipitated in a 45–55% AS cut. After centrifugation, resuspension and dialysis, the sample was purified by high pressure liquid chromatography at 294 K, first on a Pharmacia MONO Q HR 10/10 column (0–1 *M* sodium phosphate, pH 7.0/100 min, 2 ml $\mathrm{min^{-1}}$ flow rate), then on a Pharmacia Phenyl Superose HR 5/5 column (50 m*M* potassium phosphate, pH 7.0, 34–0% AS/30 min, 0.5 ml $\mathrm{min^{-1}}$), and finally on a Pharmacia MONO P HR 5/20 column (0–0.8 *M* sodium phosphate, pH 7.0/200 min at 1.5 ml $\mathrm{min^{-1}}$). The course of purification was monitored by sodium dodecyl sulfate polyacrylamide-gel electrophoresis, and fractions were assayed by the method of Houen & Leonardsen (1992), using β-phenylethylamine as substrate.

The initial crystallization conditions were screened by means of the hanging-drop method using a revised sparse-matrix method (Jancarik & Kim, 1991). The protein concentration was 6–9 mg $\mathrm{ml^{-1}}$. Form I crystals were grown by batch dialysis against 0.1 *M* cacodylate, 0.2 *M* calcium acetate and 10%(*w*/*v*) PEG 8000, pH 6.5, at 277 K (the conditions being taken from one of the successful screening trials). A tendency of the crystals to be twinned was noted, and the existence of a large super-cell was later discovered. Form II crystals were obtained using the hanging-drop method. Drops containing equal volumes of protein solution and precipitant were equilibrated against the precipitant (25% saturated ammonium sulfate, 0.1 *M* Tris, pH 8.7). Form III crystals were grown by the same method, using 0.2 *M*

Table 1. *Summary of the crystallographic characterization and data collection*

	Form I	Form II	Form III
Crystal size (μm)	400 × 250 × 50	300 × 150 × 50	1100 × 400 × 60
Symmetry	Monoclinic	Monoclinic	Orthorhombic
Space group	$C2$	$C2$	$P2_12_12_1$
Cell parameters			
a (Å)	159.0	157.6	112.2
b (Å)	65.1	64.3	162.6
c (Å)	71.2	92.6	69.4
β ( )	112.5	112.6	90
V_m (Å^3 Da^{-1})	2.41*	3.07*	2.24*
No. of subunits/ asymmetric unit	1	1	2
Resolution (Å)	3.0†	2.8†	3.0†
R_{merge}‡	—§	0.082	0.071

* A molecular weight of 70 644 Da per subunit is assumed (Tanizawa *et al.*, 1994). † Resolution is defined as the limit beyond which fewer than 50% of reflections have $I \geq 3\sigma(I)$. ‡ $R_{\text{merge}} = \sum|I(k) - \langle I\rangle|/\sum I(k)$ where $I(k)$ is the value from the kth measurement of the intensity of a reflection, $\langle I\rangle$ is the mean value of the intensity of that reflection, and the summation is over all measurements. § Crystals of form I exhibited a super-cell with symmetry $C2$ and a volume five times that of the cited cell. The diffraction pattern extended to 3 Å resolution but intensities were not recorded.

ammonium acetate, 0.1 *M* Tris, 30%(*w*/*v*) PEG 4000 at pH 8.5 as precipitant. Diffraction-quality crystals of all three forms could be harvested after three weeks.

3. X-ray analysis

Crystals were mounted in capillary tubes prior to diffraction experiments. X-ray intensity data were collected at ~294 K (i) on an in-house R-AXIS II image-plate system, using Cu $K\alpha$ radiation from a Rigaku rotating-anode generator equipped with mirror optics (Molecular Structure Corporation), and (ii) on the Sakabe Weissenberg camera (Sakabe, 1991) on beamline 6A2 at the Photon Factory, Tsukuba, Japan. The space groups were initially determined by inspection of computer graphics images of the Photon Factory image plates, and were confirmed by careful processing of these data and additional images recorded on the R-AXIS II system. The data were processed using (i) proprietary R-AXIS software, and (ii) the program *DENZO* (Otwinowski, 1993), respectively. The results are summarized in Table 1.

The preliminary crystallographic data provide independent evidence that the two subunits of PEAO are identical. In the case of the two monoclinic crystal forms, the molecular weight calculated from the amino-acid sequence, 70 644 Da per subunit, leads to reasonable values of 2.41 and 3.07 Å^{3}Da^{-1}, respectively, for the Matthews coefficient V_m (Matthews, 1968) only if the asymmetric unit of the crystals comprises one protein subunit. The conclusion that the two protein subunits are identical is consistent with the isolation and cloning of a single PEAO gene coding for 638 residues (Tanizawa *et al.*, 1994). A structure analysis is in progress.

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