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Crystallization and preliminary X-ray analysis of polyamine oxidase from *Zea mays* L.

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Abstract

Polyamine oxidase catalyses the oxidation of the secondary amino group of spermine, spermidine and their acetyl derivatives. The enzyme plays an important role in the regulation of polyamine intracellular concentration and is a member of the family of flavin-containing amine oxidases. Crystals of maize polyamine oxidase have been grown by the hanging-drop vapour-diffusion technique. The crystals are in hexagonal space group $P6_122$ (or $P6_522$) with cell dimensions $a = b = 184.6$, $c = 280.9$ Å. A native data set has been collected to 2.7 Å resolution at a synchrotron radiation source.

1. Introduction

The natural polyamines spermine and spermidine are ubiquitous polycationic metabolites which are essential for cell growth and proliferation (Heby, 1989; Tabor & Tabor, 1984). Although their function is not precisely defined, it is known that polyamines modulate the catalytic activity of several proteins such as hormone receptors, G proteins, protein kinases and DNA topoisomerase I and II (see Leroy *et al.*, 1997; Xu *et al.*, 1991; Marton & Pegg, 1995, and references therein).

The intracellular concentration of polyamines is regulated by a network of metabolic pathways which control the biosynthesis, uptake and degradation of these cationic molecules (Tabor & Tabor, 1984). The interest in polyamines and their metabolism has considerably increased with the discovery that inhibition of polyamine biosynthesis is associated with a decrease in cell proliferation, opening up the possibility of designing new antineoplastic agents (Hessels *et al.*, 1991; Chamaillard *et al.*, 1997, and references therein). In fact, several polyamine analogues, such as those with an ethyl or benzyl substituent on the terminal amino groups, are currently employed as antitumour agents (Pegg & Hu, 1995; Marton & Pegg, 1995). Furthermore, it has been reported recently that accumulation of the products of polyamine catabolism resulting from the uptake of polyamine analogues leads to programmed cell death (Hu & Pegg, 1997; Casero & Pegg, 1993; Ha *et al.*, 1997).

Polyamine oxidase (PAO) is one of the key enzymes in polyamine catabolism. PAO catalyses the oxidation of the secondary amino group of spermine, spermidine and their acetyl derivatives. The site of the enzymatic attack is found to vary depending on the source of the enzyme. Thus, animal PAO transforms spermidine and spermine into putrescine (1,4-diaminobutane) and spermidine, respectively, plus propionaldehyde and H_2O_2 (Fig. 1). Conversely, in plants and bacteria, the enzyme acts on the other side of the secondary amino group, so that the products resulting from oxidation of spermidine and spermine are 4-aminobutyraldehyde and 3-aminopropyl-4-aminobutyraldehyde, respectively (Tabor & Tabor, 1984; Federico & Angelini, 1991; Fig. 1). Despite these differences, the plant, bacterial and animal proteins have essentially identical molecular properties. PAO is a monomeric soluble enzyme with a molecular weight of about 53 kDa. The prosthetic group is flavin–adenine dinucleotide (FAD) which is non-covalently bound to the protein (Tabor & Tabor, 1984; Seiler, 1995).

The recent determination of the primary structure of maize (*Zea mays* L.) PAO has revealed that the enzyme presents a Gly–X–Gly–X–X–Gly sequence characterizing the $\beta\alpha\beta$ unit forming the FAD binding site (Wierenga *et al.*, 1983; Tavladoraki *et al.*, 1998). With the exception of this fingerprint sequence, PAO does not exhibit any sequence similarity to proteins of known three-dimensional structure. However, maize PAO is clearly homologous (sequence identity of about 20–30%) to vertebrate monoamine oxidase, a thoroughly investigated flavoenzyme which is the target of L-DOPA and selegeline, two widely used drugs against Parkinson's disease (Singer & Ramsay, 1995). PAO and monoamine oxidase differ in their substrate specificity, since PAO oxidizes secondary amines whereas monoamine oxidase acts on primary amino groups. However, the sequence similarity indicates that the two enzymes form a group of related flavin-dependent amine oxidases which are likely to share a similar mechanism of catalysis.

We have undertaken the X-ray analysis of maize PAO, in the framework of a project devoted to analysis of the functional properties of flavoenzymes (Mattevi, Fraaije *et al.*, 1997; Mattevi, Vanoni *et al.*, 1997). Compared with animal PAO, the maize protein offers the advantage that it can be purified from the plant leaves in sufficiently large quantities to carry out crystallization experiments (Federico *et al.*, 1990). Furthermore, the maize enzyme represents the first PAO of known primary structure (Tavladoraki *et al.*, 1998). We expect that the three-dimensional structure will provide insight into the determinants of substrate specificity and catalytic mechanism in the flavin-dependent amine oxidases.

2. Experimental methods

2.1. *Extraction and purification*

Maize PAO was purified by modifying a previously reported procedure (Federico *et al.*, 1989). Maize seeds (*Zea mays* L.) were soaked for 10 h in aerated tap water and grown in moistened vermiculite for 10 days in the dark at 298 K. Plants (mesocotyls, coleoptiles and leaves, 1.5 kg) were homogenized in a Waring Blendor with 4 volumes of cold 50 m*M* NaH_2PO_4 and the homogenate was filtered through Miracloth (Calbiochem). The solid residue (cell walls and fibres) was washed three times with the same buffer. The enzyme was then eluted

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from the solid residue with 1 volume of 50 m*M* NaH_2PO_4, 20% saturated with solid ammonium sulfate. The slurry was pressed through Miracloth and the extract centrifuged at 13000*g* for 30 min at 277 K. The precipitate was discarded and the supernatant was brought to 70% saturation with solid ammonium sulfate. After 2 h at 277 K, the suspension was centrifuged at 13000*g* for 30 min. The pellet was dissolved in 100 ml of 0.2 *M* NaH_2PO_4 and dialysed against 15 l of the same buffer at 277 K. After centrifugation of the dialysate, the supernatant was applied to a carboxymethyl cellulose column (CM52, Whatman; 100 ml) equilibrated with 0.2 *M* NaH_2PO_4. The column was washed with the same buffer and eluted with 0.1 *M* NaCl in 0.2 *M* NaH_2PO_4. The fractions with the highest specific activity were applied directly to a hydroxylapatite column (BioRad; 20 ml) pre-equilibrated with 0.1 *M* NaCl in 0.2 *M* NaH_2PO_4, and washed with the same buffer. The enzyme was eluted with 0.5 *M* sodium phosphate buffer (pH 6.5). The active fractions were dialysed against 50 m*M* sodium phosphate buffer (pH 6), and after centrifugation the supernatant was applied to a HiTrap SP column (Pharmacia Biotech) equilibrated with the same buffer. The protein was eluted with 0.4 *M* NaCl in 50 m*M* sodium phosphate buffer (pH 6) and collected in 3 ml fractions. Each yellow fraction was checked for purity by specific activity, visible absorbance and SDS–PAGE. The purest fractions were pooled and used for crystallization studies. On average, the amount of crystallizable protein produced per kilogram of plant cells was about 10 mg.

2.2. *Crystallization*

Crystallization conditions were screened using the hanging-drop vapour-diffusion technique. Crystals of maize PAO were obtained using ammonium sulfate as precipitant. The hanging drops were formed by mixing equal volumes of the protein and reservoir solutions, where the protein solution consisted of 5 mg ml^{-1} enzyme in 300 m*M* NaCl and 50 m*M* sodium phosphate buffer (pH 6.0), and the reservoir solution contained 100 m*M* sodium acetate buffer (pH 4.6), 0.02% NaN_3 and 48% saturated ammonium sulfate. Crystals grew as hexagonal prisms at 293 K within 7–14 days, reaching a maximum size of 0.3 × 0.3 × 0.6 mm.

For data collection, crystals were soaked for a few seconds in a solution containing 50% saturated ammonium sulfate, 30%(*v*/*v*) glycerol and 100 m*M* sodium acetate buffer (pH 4.6). The crystals were then mounted in a rayon loop (Hampton Research) supported by a brass mounting pin and quickly transferred to a liquid-nitrogen stream maintained at 100 K. A complete 2.7 Å data set was collected at the X-ray diffraction beamline of the ELETTRA Synchrotron facility (Trieste, Italy). A MAR Research detector (345 mm) was used as detector with monochromated radiation (λ = 1.0 nm). The data were integrated with *MOSFLM* (Leslie, 1992) and scaled using the program *SCALA* from the CCP4 suite (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

Crystals of maize PAO grow reproducibly at pH 4.6 using ammonium sulfate as precipitant. The low pH of the crystallization medium corresponds to the optimum pH for stability, as suggested by calorimetric studies which show that the thermal stability of the protein increases by 14 K at pH 5. Furthermore, at the pH of crystallization, the protein retains 30% of its activity with respect to the pH optimum (pH 6.5) for catalysis (Federico *et al.*, 1990).

A data set was measured at the ELETTRA synchrotron (Trieste). A total of 225738 measured intensities were merged into a set of 75905 independent reflections with an R_{merge} value of 6.3% and a completeness of 95.5% to 2.7 Å resolution. In the highest resolution shell (2.77–2.70 Å) R_{merge} is 7.5%, with 94.5% of the measured reflections greater than $3\sigma(I)$ (Table 1). From the data set, the space group was determined to be $P6_122$ (or $P6_522$) with cell dimensions $a = b = 184.6$, $c = 280.9$ Å. Considering the volume of the unit cell, several values for the number of molecules per asymmetric unit are possible. Assuming a number of molecules per asymmetric unit ranging from 3 to 6, results in a V_m parameter (Matthews, 1968) in the range 4.23–2.12 $Å^3$ Da^{-1} and a solvent content of 71–42%. In

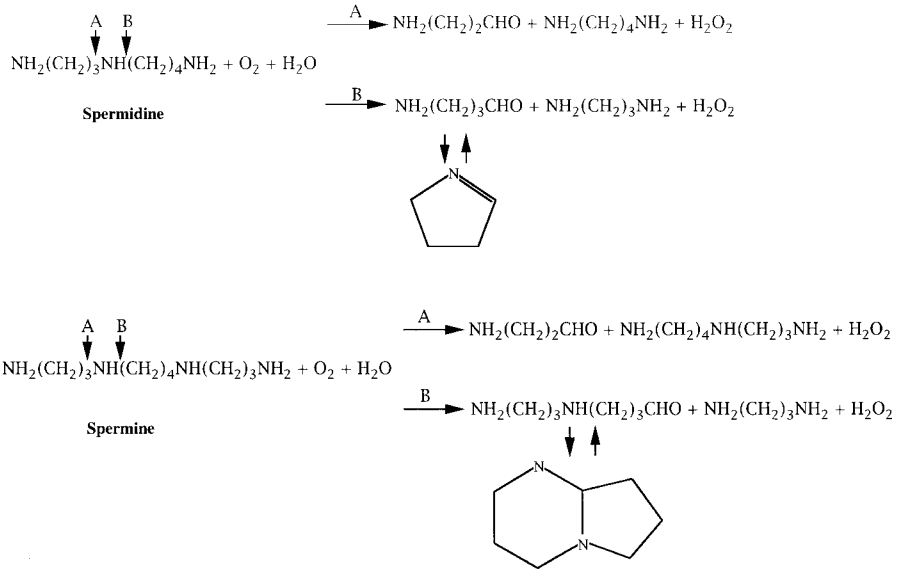

Fig. 1. Catalytic reaction and substrate specificity of PAO. A and B indicate the site of the enzymatic attack in the animal and plant enzyme, respectively. The plant enzyme oxidizes spermidine and spermine into 4-aminobutyraldehyde and 3-aminopropyl-4-aminobutyraldehyde, which spontaneously cyclize to Δ^1-pyrroline and 1,5-diazabicyclononane, respectively.

Table 1. *PAO data-collection statistics*

Resolution	$I > 3\sigma(I)$ (%)	Completeness (%)	Number of reflections	R_{merge} (%)†
99.0–7.32	99.6	88.4	3274	6.2
7.32–6.00	98.8	94.9	3169	4.9
6.00–5.18	99.1	96.0	3743	5.0
5.18–4.63	99.3	95.4	4092	5.3
4.63–4.23	99.3	96.0	4543	5.5
4.23–3.92	99.1	97.0	4993	5.7
3.92–3.76	99.2	97.4	5461	6.0
3.76–3.45	98.9	98.1	5848	6.1
3.45–3.28	98.2	98.6	6200	6.4
3.28–3.12	97.6	99.0	6536	6.3
3.12–3.00	97.2	99.4	6850	6.7
3.00–2.87	96.1	99.1	7124	7.1
2.87–2.77	96.0	99.4	7404	7.4
2.77–2.70	94.5	86.5	6668	7.5
Overall	97.3	95.5	75905	6.3

† $R_{merge} = \sum_h \sum_i |I_{ih} - \langle I_h \rangle| / \sum_h \sum_i I_{ih}$ where $\langle I_h \rangle$ is the mean intensity of the i observations of reflection h.

order to clarify this problem, self-rotation function calculations were performed by means of the programs *AMoRe* (Navaza, 1994) and *GLRF* (Tong & Rossmann, 1990). The calculations were carried out using data at different resolution limits and several values for the integration radius. However, none of the calculated self-rotation functions produced clear peaks. Similarly, a Patterson calculated with the native structure-factor amplitudes did not display any significant peak. Therefore, no firm conclusions as to the number of molecules in the asymmetric unit could be gathered from these calculations.

For structure determination, the method of multiple isomorphous replacement will be employed. A search for heavy-atom derivatives is currently in progress.

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