crystallization communications

Crystallization and preliminary X-ray diffraction analysis of maize aldose reductase

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Maize aldose reductase (AR) is a member of the aldo-keto reductase superfamily. In contrast to human AR, maize AR seems to prefer the conversion of sorbitol into glucose. The apoenzyme was crystallized in space group $P2_12_12_1$, with unit-cell parameters $a = 47.2$, $b = 54.5$, $c = 100.6$ Å and one molecule in the asymmetric unit. Synchrotron X-ray diffraction data were collected and a final resolution limit of 2.0 Å was obtained after data reduction. Phasing was carried out by an automated molecular-replacement procedure and structural refinement is currently in progress. The refined structure is expected to shed light on the functional/enzymatic mechanism and the unusual activities of maize AR.

1. Introduction

Proteins of the aldo-keto reductase (AKR) superfamily are monomeric $(\alpha/\beta)_8$-barrel proteins of approximately 35 kDa in molecular weight which use NAD(P)(H) to catalyze the reduction of aldehydes and ketones, monosaccharides, ketosteroids, protaglandins and other specific substrates (Jez & Penning, 2001). AKRs occur in prokaryotes and eukaryotes, including yeast, plant, amphibia and mammals (Jez *et al.*, 1997). Aldose reductase (AR; EC 1.1.1.21) is a member of the AKR superfamily (Bohren *et al.*, 1989) that has a monomeric NADPH-dependent oxidoreductase activity with broad substrate specificity. In some species, AR is able to catalyze the reduction of glucose to sorbitol in the polyol pathway (Jez *et al.*, 1997; Ueda *et al.*, 2004). This pathway is especially relevant to diabetic patients. Hyperglycaemia causes an increased flux of glucose through the polyol pathway, which elicits various metabolic imbalances and early damage to tissues that undergo insulin-independent uptake of glucose, such as the lens, retina, peripheral nerve and renal glomerulus. For this reason, AR inhibition has been considered to be an attractive approach to halting long-term diabetic complications (Srivastava *et al.*, 2005). Although the structure of AR has been extensively studied in humans (Rondeau *et al.*, 1992; Wilson *et al.*, 1992; Biadene *et al.*, 2007), little is known about ARs in plants. Some evidence suggests that plant ARs are involved in abiotic stress resistance. For example, freezing treatment induces AR expression in brome grass (Lee & Chen, 1993) and in *Xerophyta viscose* AR is expressed under dehydration conditions (Mundree *et al.*, 2000).

Barley AR is expressed in embryos with a pattern that correlates with the seed-maturation phase (Bartels *et al.*, 1991; Roncarati *et al.*, 1995). Likewise, rice AR is expressed at high levels when the seeds reach maturity (Sree *et al.*, 2000). In maize, on the other hand, AR is broadly expressed but is apparently more abundant in embryos, especially in the earlier phases of development. Maize AR not only displays the typical activity of AKR-family members, but is also able to convert sorbitol into glucose, an activity which seems especially adapted to maize seeds and allows developing maize embryos to metabolize substantial amounts of sorbitol from the endosperm (de Sousa *et al.*, work to be published). We have initiated a study that aims to understand the apparent preference of maize AR for sorbitol over glucose as a substrate, a feature that is the opposite of that observed in humans and fungi. Here, we report the crystallization of
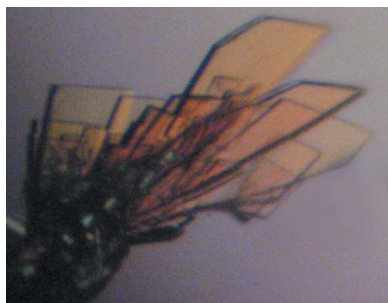
apo maize aldose reductase and its preliminary crystallographic analysis.

2. Material and methods

2.1. Cloning, expression and purification

Detailed methodology will be published elsewhere (de Sousa *et al.*, work to be published). The gene encoding maize AR (DQ517521) was amplified and cloned into the vector pET28a (Novagen). Competent *Escherichia coli* strain BL21(DE3) pRil cells were transformed with positive recombinant plasmid and cultured in LB broth with 50 µg ml^{-1} kanamicin and 100 µg ml^{-1} chloramphenicol. Lactose (100 mM) was added to induce protein expression and incubation proceeded for an additional 6 h. The cells were harvested and pellets were resuspended in affinity buffer (50 mM sodium phosphate buffer pH 7.2) containing 100 mM NaCl and 5% glycerol. Lysozyme was added to a final concentration of 1.0 mg ml^{-1}. The suspension was incubated for 1 h at 277 K and sonicated. Insoluble debris was removed by centrifugation and the clarified supernatant was used for protein purification by IMAC (immobilized metal-affinity chromatography). The eluted maize AR was dialysed in anion-exchange buffer (20 mM Tris–HCl pH 7.5, 5 mM EDTA, 7 mM β-mercaptoethanol and 20 mM NaCl) and then purified on a Q-Sepharose FF anion-exchange chromatography column (1 ml; Amersham Biosciences, USA) using an ÄKTA FPLC system (Amersham Biosciences, USA). Bound proteins were eluted using an NaCl gradient. Protein concentration and purity was analyzed by SDS–PAGE. More accurate estimations for purified maize AR were made based on the absorbance at 280 nm, using a calculated extinction coefficient of 1.824 g l^{-1} cm^{-1} (Pace & Schmid, 1997).

2.2. Crystallization

Initial crystallization conditions were screened in Tissue Culture Test Plates 24 (TPP) by the hanging-drop method at 293 K, using the sparse-matrix method (Jancarik & Kim, 1991) implemented in the Crystallization Basic and Extension Kits for Proteins (Sigma). Imperfect crystals were obtained in various conditions and were used as a guide for further optimization. Good diffracting crystals were obtained in a condition similar to condition 22 of the Crystallization Basic Kit. The optimum reservoir solution, consisting of 26% PEG 4000 (Sigma/Fluka), 0.2 M sodium acetate (Vetec) and 0.1 M Tris–HCl pH 6.5 (Vetec), was mixed with protein solution (10 mg ml^{-1} in water) in equal amounts and equilibrated against reservoir solution.
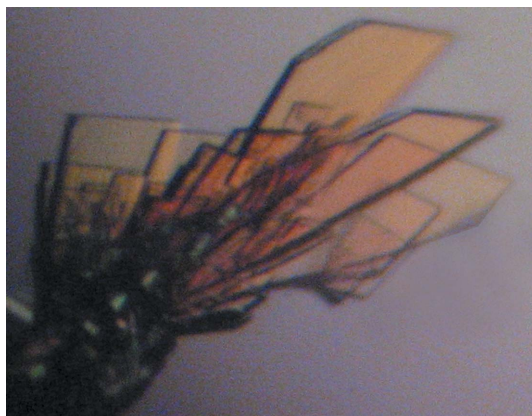

Figure 1
Representative crystals of maize AR grown as clusters of plates with maximum dimensions of approximately 400 × 200 × 50 µm.

Table 1
Crystal parameters and data-collection statistics.

Values in parentheses are for the last resolution shell.

Space group	$P2_12_12_1$
Unit-cell parameters (Å)	
a	47.2
b	54.5
c	100.6
Solvent content (%)	34.4
Molecules in ASU	1
Resolution range (Å)	50.32–2.00 (2.11–2.00)
No. of images	129
No. of observed reflections	77147 (11331)
No. of rejected reflections	2592
No. of unique reflections	18151 (2593)
Multiplicity	4.3 (4.4)
Completeness (%)	99.8 (99.8)
R_{sym} (%)	11.8 (38.5)
$\langle I/\sigma(I) \rangle$	9.7 (2.8)
Wilson plot B factor (Å^2)	24.3

Crystals were obtained as clusters of plates and grew to full size in two weeks at 293 K (Fig. 1). Attempts to obtain single crystals by the use of additives, seeding and other strategies were not succesful. However, single plates manually separated from the initial clusters exhibited good morphology and size and proved to be of sufficient quality for data collection.

2.3. Data collection and processing

Cryocrystallographic techniques (Garman & Schneider, 1997) were employed to prevent radiation damage. Crystals were briefly soaked in a cryoprotectant solution containing 15%(v/v) ethylene glycol and were rapidly frozen in a gaseous nitrogen stream at 100 K (Oxford Cryosystems). Data were collected by the oscillation method on beamline D03B-MX1 at the Laboratório Nacional de Luz Síncrotron (LNLS, Campinas-SP, Brazil; Polikarpov *et al.*, 1998) using a MAR CCD 165 detector. The X-ray wavelength was 1.425 Å with a crystal-to-detector distance of 78.2 mm, giving an outer-edge resolution of 1.8 Å. The oscillation range was 1° and the exposure time per image was 240 s. Data reduction was performed with the packages *MOSFLM* and *SCALA* (Collaborative Computational Project, Number 4, 1994; Winn *et al.*, 1997). A summary of crystal parameters and data-collection statistics is presented in Table 1.

3. Results and discussion

Initial attempts were made to solve the crystal structure of maize AR using homologous protein structures available in the Protein Data Bank. The program *MATTHEWS_COEF* (Collaborative Computational Project, Number 4, 1994; Winn *et al.*, 1997) was used to calculate the Matthews coefficient (Matthews, 1968) and the Matthews probability (Kantardjieff & Rupp, 2003). Across all resolution ranges, including the high-resolution limit, the probability of $V_M = 1.9$ Å^3 Da^{-1} and a solvent content of 34.4% was estimated to be 1.0, unequivocally indicating the presence of one molecule in the asymmetric unit. Primary sequence searches and sequence alignments were made with ENTREZ and *BLAST* (Altschul *et al.*, 1997). Molecular-replacement trials were performed manually using the program *AMoRe* (Navaza, 1994; Winn *et al.*, 1997) and although there was an indication of possible solutions, the low contrast obtained suggested the application of automated methods.

Accordingly, the automated procedure for molecular replacement implemented in the program *MrBUMP* (Keegan & Winn, 2007) was adopted. *MrBUMP* employed the programs *FASTA* (Pearson &

Lipman, 1988) and *CLUSTALW* (Chenna *et al.*, 2003) for the template search, *MOLREP* (Vagin & Teplyakov, 1997; Winn *et al.*, 1997) for molecular replacement and *REFMAC* (Murshudov *et al.*, 1997; Winn *et al.*, 1997) for initial evaluation of the possible solutions. The most sucessful model was prepared with *CHAINSAW* (Schwarzenbacher *et al.*, 2004; Winn *et al.*, 1997) from an homologous structure of pig aldose reductase complexed with sorbinil (PDB code 1ah0; Urzhumtsev *et al.*, 1997), the 11th hit in the primary *BLAST* search (48% sequence identity, corresponding to 138 of 285 amino-acid residues). After 30 cycles of automated restrained refinement, the R factor and R_{free} (5% of the total reflections) were 37.9% and 43.3%, respectively.

The low contrast in the solutions obtained from the manual molecular-replacement trials and the current relatively high value of R_{free} seem to be an indication that there are important differences in the three-dimensional structures of maize AR and other members of the AKR family, which could be related to its diverse properties as mentioned above. Refinement is in progress and the structural details will be reported in due course.

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