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Crystallization and preliminary X-ray analysis of an alditol oxidase from *Streptomyces coelicolor* A3(2)

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Alditol oxidase is a 45 kDa enzyme containing a covalently bound FAD cofactor. This oxidase efficiently oxidizes a range of alditols to the corresponding aldoses. Owing to its substrate range and regioselectivity, this enzyme is an interesting candidate for biotechnological applications. Crystals of alditol oxidase from *Streptomyces coelicolor* A3(2) were obtained by the hanging-drop vapour-diffusion method and diffracted to 1.1 Å resolution. The crystals belong to space group *C*2, with unit-cell parameters $a = 107$, $b = 68$, $c = 58$ Å, $\beta = 94^\circ$. Crystals of seleno-L-methionine-labelled alditol oxidase were obtained after seeding the crystallization drops with native microcrystals and showed a diffraction limit of 2.4 Å.

1. Introduction

Owing to their broad substrate specificity and exquisite regioselectivity, polyol oxidases are an interesting class of biocatalysts for analytical and synthetic purposes. For this reason, glucose oxidase and galactose oxidase, both of fungal origin, can be found in a multitude of biotechnological applications. Chemical methods cannot compete with the selectivity with which these enzymes oxidize polyols. However, to date only a few oxidases acting on polyols have been identified, narrowing the biocatalytic exploitation of this important class of oxidative enzymes. Most known polyol oxidases contain a flavin cofactor to perform catalysis and belong to a specific family of flavoproteins: the vanillyl-alcohol oxidase (VAO) family (Fraaije *et al.*, 1998). A special feature of VAO-type flavoproteins is the fact that many members of this family contain an FAD flavin cofactor that is covalently linked to a histidine residue. The target histidine to which the FAD cofactor is attached can be predicted using a sequence-motif search.

Using a genome-mining approach, we have recently identified a VAO homologue that is active on a specific class of polyols: alditols (Heuts *et al.*, 2007). This alditol oxidase (AldO) is present in the proteome of *Streptomyces coelicolor* and contains a histidyl-bound FAD. Although the physiological function of this oxidase is as yet unknown, AldO is a promising candidate for biocatalytic exploitation in the fields of biosynthesis and biosensors. Biochemical investigation has shown that AldO performs selective oxidation of only one of the terminal primary hydroxyl groups of aliphatic alditols (Fig. 1). While xylitol (five C atoms) and sorbitol (six C atoms) are the best substrates, L-threitol (four C atoms) and D-mannitol (six C atoms) are poorer substrates, as can be seen from their relatively high K_m values (Heuts *et al.*, 2007). This illustrates that the enzyme is able to distinguish between structurally related polyols.

Here, we report the overexpression and purification of recombinant AldO and the first diffraction data collected from native and seleno-L-methionine-labelled protein crystals. Elucidation of the crystal structure of AldO will provide insight into the mechanism of AldO-mediated regioselective oxidation reactions and its substrate specificity.

2. Protein overexpression and purification

Escherichia coli TOP10 competent cells, the TOPO TA cloning kit for sequencing and the pBAD/Myc-His A vector were obtained from

Invitrogen. Using *Pfu* DNA polymerase, the *AldO* gene was amplified from *S. coelicolor* A3(2) genomic DNA. For cloning, the following primers were used: *XOmbp*_fw (5′-CTCGAATTCATGAGCGACATCACGGTC) and *XOmbp*_rv (5′-TATCTGCAGTCAGCCCGCGAGCACCCC). The amplified *AldO* gene was ligated into the TOPO vector. The resulting TOPO-AldO clone was digested with *Eco*RI and *Pst*I, after which the *AldO* gene was isolated from an agarose gel. The *AldO* gene was subsequently ligated into a digested pBAD-MBP vector, yielding the pBAD-MBP-AldO plasmid.

E. coli TOP10 cells were transformed with pBAD-MBP-AldO and grown in Terrific broth medium supplemented with 50 µg ml^{-1} ampicillin to an OD_{600} of 0.8 and then induced with 0.02%(*w*/*v*) L-arabinose at 290 K for 3 d. This yielded cells containing overexpressed AldO fused with maltose-binding protein (MBP-AldO). Cells from 1.5 l culture broth were collected by centrifugation at 4000*g* at 277 K for 15 min and resuspended in 50 m*M* potassium phosphate buffer pH 7.5. The cells were disrupted by sonication and subsequent centrifugation at 23 000*g* and 277 K for 30 min to remove cell debris. The supernatant was loaded onto a Q-Sepharose column (Amersham Biosciences) and the column was washed until the A_{280} reached a value of lower than 0.05. MBP-AldO was eluted by applying a 0–1 *M* KCl linear gradient. The fractions containing AldO were easily identified from the bright yellow colour of the flavin cofactor covalently bound to the enzyme. These yellow fractions were pooled and concentrated using an Amicon stirred cell fitted with a YM-30 membrane (Millipore). Finally, the concentrated enzyme solution was loaded onto a HiPrep 26/10 Desalting Column (Amersham Biosciences) to remove KCl. This purification procedure yielded an impressive 350 mg MBP-AldO from 1 l culture broth.

During SDS–PAGE, purified MBP-AldO runs as a single band at about 87 kDa, which is in agreement with the calculated weight of 88.1 kDa. The MBP tag could be removed by treating MBP-AldO with modified trypsin lacking chymotrypsin activity (Promega). To perform this, 70 µ*M* MBP-AldO was incubated with 0.8 µg ml^{-1} modified trypsin at 296 K for 1.5 h. This yielded the two separate proteins MBP and AldO. MBP could effectively be removed by applying the mixture onto amylose resin (New England Biolabs). The final protein quality was assessed by SDS–PAGE and UV–Vis spectroscopic analysis. It was anticipated that tryspin digestion would yield native AldO with an extension of four amino acids at the N-terminus (Ile-Ser-Glu-Phe). Mass-spectrometric analysis revealed that trypsin digestion had indeed occurred at the expected proteolytic cleavage site. The determined weight of the extended native AldO of 45 665 Da corresponds well to the calculated weight of 45 663 Da.

Table 1
Data-collection statistics for native and SeMet AldO.

Data were collected at beamlines ID23-EH2 (native) and ID23-EH1 (SeMet) at the ESRF. Values in parentheses are for the highest resolution shell.

	Native	SeMet
Wavelength (Å)	0.8730	0.9761
Resolution (Å)	24.0–1.1 (1.16–1.10)	20.0–2.4 (2.53–2.40)
Space group	*C*2	*C*2
Unit-cell parameters (Å, °)	*a* = 106.0, *b* = 68.6, *c* = 58.0, *β* = 95.1	*a* = 106.2, *b* = 68.8, *c* = 58.6, *β* = 95.3
V_M (Å^3 Da^{-1})	2.21	2.21
Total measurements	605996 (86778)	114799 (17152)
Unique reflections	167187 (24377)	16448 (2394)
Average redundancy	3.6 (3.6)	7.0 (7.2)
$I/\sigma(I)$	7.6 (2.6)	21.1 (16.0)
Completeness (%)	100.0 (100.0)	100.0 (100.0)
Anomalous completeness† (%)	—	99.7 (99.6)
Anomalous redundancy	—	3.6 (3.6)
R_{merge}‡	0.157 (0.455)	0.086 (0.124)

† Completeness calculations treat Friedel pairs as separate observations. ‡ $R_{merge} = \sum_{\mathbf{h}} \sum_i |I(\mathbf{h})_i - \langle I(\mathbf{h})\rangle| / \sum_{\mathbf{h}} \sum_i I(\mathbf{h})$, where $I(\mathbf{h})_i$ is the scaled observed intensity of the *i*th symmetry-related observation of reflection **h** and $\langle I(\mathbf{h})\rangle$ is the average intensity.

Seleno-L-methionine labelling was achieved by inhibition of methionine biosynthesis (Doublié, 1997). *E. coli* BL21 DE3 cells were transformed with the pBAD-MBP-AldO plasmid, grown overnight in Luria–Bertani broth supplemented with the appropriate antibiotics, pelleted and resuspended in M9 broth. The suspension was used to inoculate 1 l of the same medium at a 1/30 dilution. This culture was grown at 310 K to mid-log phase (OD = 0.5). At this stage, 100 mg l^{-1} amino acids (lysine, phenylalanine, leucine, isoleucine, proline, valine and threonine) were added. The culture was then supplemented with 100 mg l^{-1} seleno-L-methionine and allowed to cool to 290 K before overnight induction with 0.2%(*w*/*v*) arabinose. The SeMet-labelled protein showed purification properties identical to those of the native enzyme. Using this seleno-L-methionine culture broth, the yield was very poor compared with that of the wild-type enzyme: the typical yield was 4–5 mg MBP-AldO from 1 l culture broth.

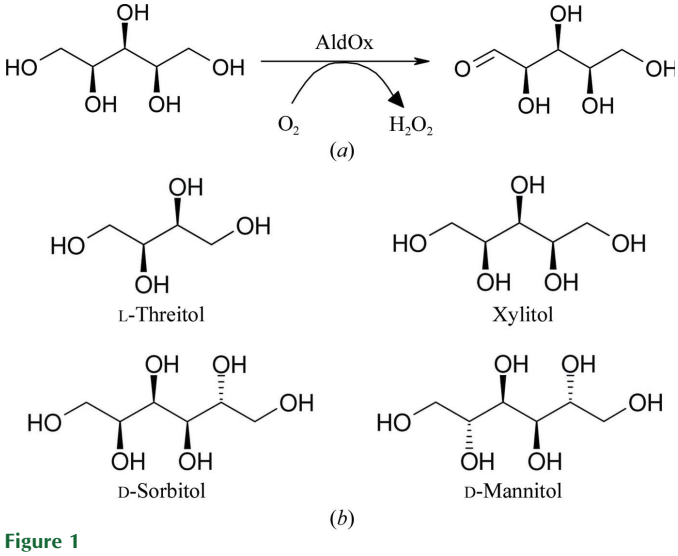
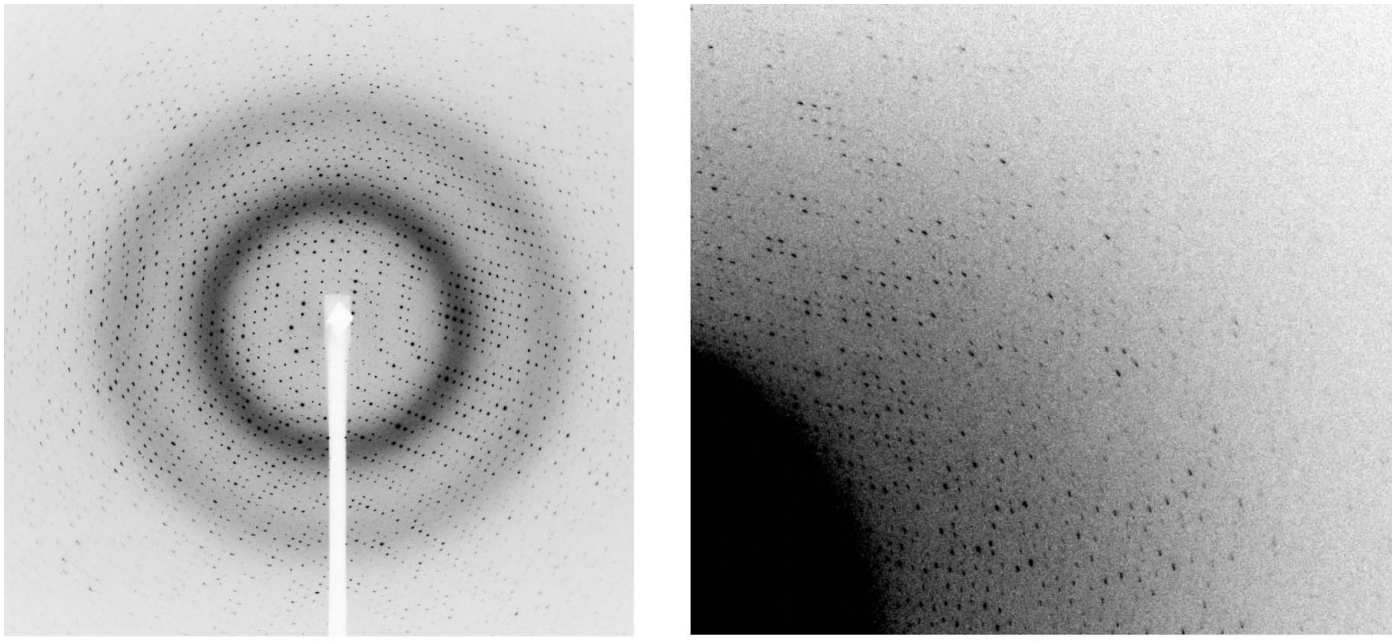


Figure 1
(*a*) Reaction catalyzed by AldO with xylitol substrate and (*b*) the chemical formulae of other enzyme substrates. The other substrates are AldO substrates, like xylitol, but are oxidized more slowly.

Figure 2
Crystals of native AldO.



(*a*) (*b*)

Figure 3
(*a*) A typical diffraction image of an AldO crystal collected on beamline ID23-EH2 at the ESRF. (*b*) Enlargement showing detail of the diffraction spots in the highest resolution shell (1 Å).

3. Protein crystallization

Purified AldO was concentrated to about 14 mg ml^{-1} in 50 m*M* potassium phosphate buffer pH 7.5. Crystallization screening was carried out at 277 K using the hanging-drop vapour-diffusion method (Ducruix & Giegé, 1992). Sigma–Aldrich Crystallization Screen Basic and Crystallization Screen Extended were selected for initial screening. 2 µl protein solution was mixed with 2 µl reservoir solution and equilibrated against 400 µl reservoir solution. Yellow crystals appeared under more than ten different conditions after 24–36 h. Optimization of the conditions from the initial screen that produced crystals with promising diffraction properties gave the following optimal conditions: 0.1 *M* MES–HCl pH 6.5, 0.2 *M* $MgCl_2$ and 18–20%(*w*/*v*) PEG 4000. The yellow colour of the crystals arises from the presence of the covalently bound FAD cofactor (Fig. 2). The volumes of protein and reservoir solutions and the protein concentration in the final crystallization conditions were similar to those used in the primary screening experiments. The crystal dimensions are about 0.5 × 0.3 × 0.3 mm.

Seleno-L-methionine-labelled crystals of AldO grew as thin fragile plates (typical dimensions 0.2 × 0.2 × 0.1 mm) using the same crystallization condition as for wild-type protein after seeding the crystallization drop with microcrystals of native AldO (Stura & Wilson, 1992). Without seeding, it was impossible to obtain crystals suitable for X-ray analysis.

4. Data collection and X-ray analysis

Crystals were harvested from the crystallization drops, soaked in mother liquor containing 15%(*v*/*v*) glycerol for 30 s, flash-cooled and maintained at 100 K using liquid nitrogen during data collection. The quality of the diffraction was checked using a home-source X-ray generator and an R-AXIS IV detector (Rigaku MSC). A complete data set was collected to 1.1 Å resolution at the ID23-EH2 beamline at the European Synchrotron Radiation Facility in Grenoble (ESRF) using an ADSC detector. A typical diffraction image is shown in Fig. 3. The crystal-to-detector distance was set to 85 mm. 180 non-overlapping oscillation images were collected with 1° oscillation per frame and an exposure time of 1 s per frame. Data were indexed using *MOSFLM* (Leslie, 1999) and scaled and merged using *SCALA* (Collaborative Computational Project, Number 4, 1994). Data-collection and processing statistics are shown in Table 1. The calculated Matthews coefficient suggests the presence of one molecule in the asymmetric unit, with a solvent content of around 40% (Matthews, 1968). A single-wavelength anomalous diffraction (SAD) data set was collected from a selenomethionyl crystal at a wavelength of 0.9761 Å, with an anomalous completeness of 99.7% in the last resolution shell (2.4 Å; Table 1). We are currently in the process of solving the three-dimensional structure of AldO using SAD phasing.

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