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Crystallization of the FAD-independent acetolactate synthase of *Klebsiella pneumoniae*

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Leucine and valine are formed in a common pathway from pyruvate in which the first intermediate is 2-acetolactate. In some bacteria, this compound also has a catabolic fate as the starting point for the butanediol fermentation. The enzyme (EC 4.1.3.18) that forms 2-acetolactate is known as either acetohydroxyacid synthase (AHAS) or acetolactate synthase (ALS), with the latter name preferred for the catabolic enzyme. A significant difference between AHAS and ALS is that the former requires FAD for catalytic activity, although the reason for this requirement is not well understood. Both enzymes require the cofactor thiamine diphosphate. Here, the crystallization and preliminary X-ray diffraction analysis of the *Klebsiella pneumoniae* ALS is reported. Data to 2.6 Å resolution have been collected at 100 K using a rotating-anode generator and an R-AXIS IV++ detector. Crystals have unit-cell parameters $a = 137.4$, $b = 143.9$, $c = 134.4$ Å, $\alpha = 90$, $\beta = 108.4$, $\gamma = 90^\circ$ and belong to space group $C2$. Preliminary analysis indicates that there are four monomers located in each asymmetric unit.

1. Introduction

2-Acetolactate is the precursor of the branched-chain amino acids valine and leucine. The biosynthetic pathway for these amino acids is widely distributed in plants, fungi, algae and bacteria. In some bacteria, 2-acetolactate also has another entirely different fate in butanediol fermentation. Decarboxylation of 2-acetolactate yields acetoin, while oxidative decarboxylation yields diacetyl; the latter can be reduced to acetoin then further reduced to butanediol. Both acetoin and diacetyl are important flavour components in fermented dairy products (Curic *et al.*, 1999) and in beer (Dulieu *et al.*, 2000).

The formation of 2-acetolactate involves the decarboxylation of pyruvate followed by condensation with a second molecule of pyruvate. The enzyme that catalyzes this reaction (EC 4.1.3.18; reviewed by Duggleby & Pang, 2000) is known as either acetohydroxyacid synthase (AHAS) or acetolactate synthase (ALS). In common with many other enzymes that cleave a C—C bond adjacent to a carbonyl group, AHAS/ALS uses thiamine diphosphate (ThDP) as a cofactor. Unexpectedly, the enzyme from many species (Störmer & Umbarger, 1964; Schloss *et al.*, 1985; Chang & Duggleby, 1997; Pang & Duggleby, 1999) also requires flavin adenine dinucleotide (FAD) as a cofactor. However, this is not a universal requirement and Störmer (1968) showed clearly that the enzyme from *Aerobacter aerogenes* (now *Klebsiella aerogenes*) is FAD-independent. It now seems apparent that the FAD-dependent enzyme produces 2-acetolactate for anabolic branched-chain amino-acid biosynthesis, while the FAD-independent form supplies the precursor for catabolic butanediol formation. Consistent with this distinction is the observation that the FAD-dependent enzyme can also combine pyruvate with 2-ketobutyrate to give 2-aceto-2-hydroxybutyrate, the precursor of isoleucine, while the FAD-independent form is specific for 2-acetolactate synthesis. A further difference between the two forms is that the anabolic enzyme contains both catalytic and regulatory subunits (Squires *et al.*, 1983; Pang & Duggleby, 1999; Lee & Duggleby, 2001), while the catabolic form lacks a regulatory subunit. The regulatory subunit confers upon the anabolic enzyme sensitivity to feedback regulation by branched-chain amino acids (Vyazmensky *et al.*, 1996; Pang & Duggleby, 1999; Lee & Duggleby, 2001). To reinforce the distinction between the two types of enzyme, it has been proposed (Gollop *et al.*, 1989) that the name ALS is reserved for the catabolic FAD-independent enzyme, while AHAS refers to the anabolic enzyme that requires FAD. Notwithstanding the different cofactor requirements, subunit structure, sensitivity to inhibition by branched-chain amino acids and substrate specificity of ALS and AHAS, the two proteins are clearly related and show substantial sequence similarities.

Recently, we reported the crystallization (Pang *et al.*, 2001) and structure determination (Pang *et al.*, 2002) of the catalytic subunit of *Saccharomyces cerevisiae* AHAS. This first experimental AHAS structure defines the position of FAD near the active site, but offers no obvious clue as to why there is an absolute requirement for this cofactor. To further investigate the role of FAD, we have now crystallized the FAD-independent ALS from *K. pneumoniae* and commenced structure determination.

2. Materials and methods

2.1. Expression, purification and preparation

The *ilvK* gene encoding the FAD-independent ALS from *K. pneumoniae* was isolated from the plasmid pTL8 (Peng *et al.*, 1992). The gene was amplified by PCR and cloned into the pET30a(+) vector, giving the expression plasmid pET.ilvk. The recombinant ALS expressed from this plasmid has a deduced molecular weight of 61.3 kDa. The protein possesses a native N-terminal sequence but has seven additional C-terminal residues: glutamate followed by a hexahistidine sequence.

The expression plasmid was used to transform *E. coli* strain BL21(DE3) cells and transformants were selected and grown at 310 K in Luria–Bertani medium containing 50 µg ml^{-1} kanamycin. Recombinant ALS production was induced at 295 K with 0.5 m*M* IPTG and cells were harvested after 4 h as described previously for yeast AHAS (Pang & Duggleby, 1999). Cell lysis and purification of the expressed hexahistidine-tagged protein by immobilized metal-affinity chromatography was as described previously (Pang & Duggleby, 1999), except that no FAD was added to the purification buffers. Eluted fractions containing ALS were pooled and concentrated by precipitation with 65% saturated ammonium sulfate. The protein pellet (10 mg) was resuspended in 2.5 ml storage buffer (50 m*M* potassium phosphate pH 7.0 containing 1 m*M* ThDP, 1 m*M* $MgCl_2$ and 1 m*M* DTT) and insoluble protein was removed by centrifugation. The clear ALS solution was desalted using a Pharmacia PD-10 column equilibrated with storage buffer. The desalted protein solution was concentrated by ultrafiltration and was further purified using a Superdex HR10/30 size-exclusion FPLC column. Purified ALS was eluted using storage buffer. For long-term storage and crystallization experiments, the protein solution was concentrated to about 9 mg ml^{-1}, divided into small aliquots and stored at 203 K. The purified enzyme appears to be homogeneous by SDS–PAGE and has a specific activity of 150 U mg^{-1}.

Table 1
Data-collection statistics for *K. pneumoniae* ALS.

Values in parentheses are statistics for the 2.70–2.60 Å resolution shell.

Temperature (K)	100
Resolution range (Å)	100.0–2.60
Total No. of observations [$I > \sigma(I)$]	221772 (17751)
No. of unique reflections [$I > \sigma(I)$]	71896 (6680)
Completeness (%)	93.8 (87.3)
R_{sym}†	0.073 (0.157)
$\langle I\rangle/\langle\sigma(I)\rangle$	16.5 (5.3)

† $R_{sym} = \sum|I - \langle I\rangle|/\sum\langle I\rangle$, where I is the intensity of an individual measurement of each reflection and $\langle I\rangle$ is the mean intensity of that reflection.

2.2. Crystallization and data collection

Crystallization trials were performed using the hanging-drop vapour-diffusion method at 290 K. Prior to each crystallization experiment, DTT was added to the freshly thawed enzyme aliquot to a final concentration of 5 m*M*. The search for suitable crystallization conditions was carried out using Hampton Crystal Screens I and II. Nine of the conditions tested yielded protein crystals. Preliminary X-ray diffraction experiments showed that some of the crystal forms, particularly those grown in the presence of high concentrations of organic solvents, diffracted in the range 3–4 Å, had a large unit cell and were unstable. Crystallization using PEG 8000 and ethylene glycol as the precipitants was selected for optimization by the grid-screen approach (Bergfors, 1999). Optimal crystal growth was obtained by combining 1 µl of ALS solution with 0.5 or 1 µl of a reservoir solution consisting of 0.1 *M* HEPES pH 7.5–7.7, 6–8%(*w*/*v*) PEG 8000 and 6–9%(*v*/*v*) ethylene glycol. Protein crystals as needles (Fig. 1*a*) or plate clusters (Fig. 1*b*) were visible after 1 d of incubation but took one to two weeks to reach their maximum size. Well formed plate-like rhombohedral prisms were chosen for the X-ray diffraction experiments.

Preliminary X-ray studies with these crystals at room temperature using a rotating-anode generator showed diffraction to about 2.9 Å resolution. However, the diffraction intensity of the crystals decreased

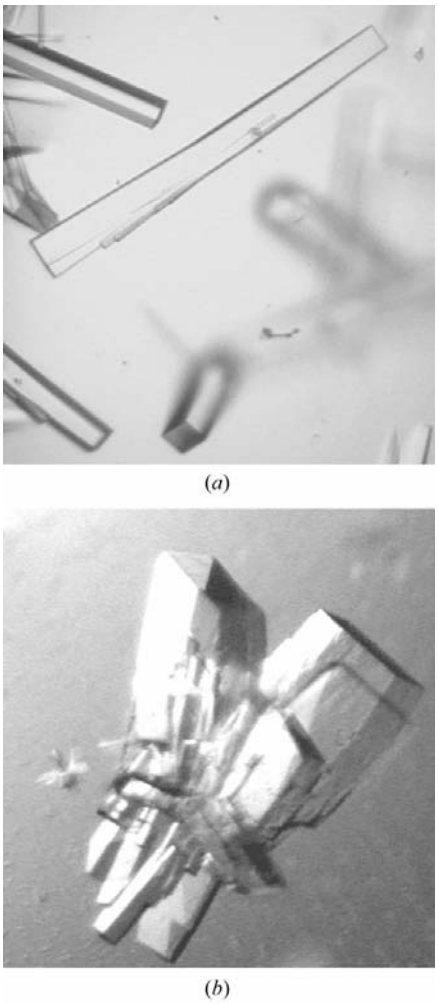

Figure 1
Crystals of *K. pneumoniae* ALS. (*a*) Needles; (*b*) plates.

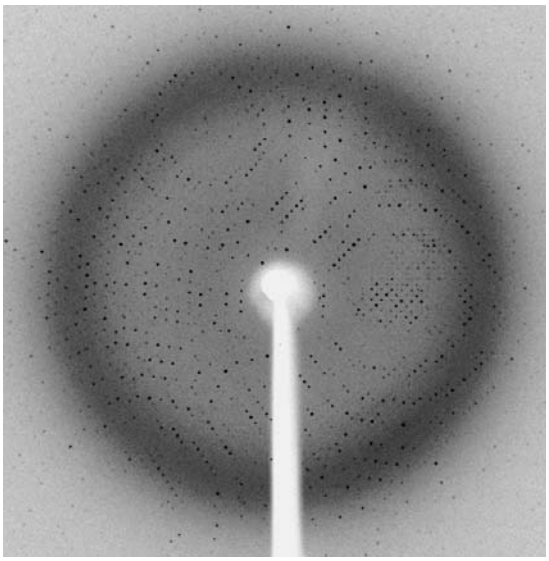

Figure 2
A 0.5° oscillation frame of a cryocooled crystal of *K. pneumoniae* ALS. Diffraction data are observed to better than 2.6 Å resolution.

quickly after the initial exposure to X-rays and a cryocooling strategy was developed where crystals were transferred to 5 µl of reservoir solution that had 30%(*v*/*v*) PEG 600 added for cryoprotection. After the transfer, the solution containing the crystals was set up as a hanging drop in a vapour-diffusion chamber with 1 ml of the hanging-drop solution used as the well solution. The crystals were then allowed to dehydrate in the sealed chamber for 24 h prior to cryocooling. The combination of dehydration and cryocooling of these crystals in PEG 600 appears to improve the resolution by up to 0.3 Å.

A complete data set from these ALS crystals was collected using an R-AXIS IV++ detector and an RU-200 rotating-anode generator operated at 50 kV and 100 mA (Fig. 2). Data images were recorded with the detector positioned 220 mm from the crystal. Each oscillation frame was 0.5° in width and was exposed for 180 s. The diffraction data were processed to 2.6 Å resolution using *DENZO* (Otwinowski & Minor, 1997) and were scaled with *SCALEPACK* (Otwinowski & Minor, 1997). Averaging of equivalent intensities allowed characterization of the Laue symmetry.

3. Results and discussion

The largest crystals of ALS have dimensions of 0.1 × 0.1 × 0.08 mm, diffract to 2.6 Å resolution (Fig. 2), have unit-cell parameters a = 137.4, b = 143.9, c = 134.4 Å, α = 90, β = 108.4, γ = 90° and belong to space group $C2$. The mosaic spread of these crystals is 0.40° at 100 K. Assuming that there are two dimers or one tetramer in the asymmetric unit, the Matthews coefficient (Matthews, 1968) V_M is 2.50 Å^3 Da^{-1}. Based on a value of 0.74 cm^3 g^{-1} for the partial specific volume of the protein, the calculated solvent content is 51.7%(*v*/*v*). Both the Matthews coefficient and solvent content are near the midpoint of the normal range expected for protein crystals (Matthews, 1968). An excellent data set to 2.6 Å resolution that has an overall completeness of 93.8% and an R_{sym} of 0.073 (Table 1) has been obtained. We are now attempting to solve the structure using molecular-replacement techniques. Using the catalytic subunit of yeast AHAS (Pang *et al.*, 2002) as the search model, we have obtained a solution with an R_{free} value of 0.49 at 2.6 Å after rigid-body refinement.

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