

# Purification, crystallization and preliminary X-ray crystallographic studies on acetolactate decarboxylase

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Acetolactate decarboxylase has the unique ability to decarboxylate both enantiomers of acetolactate to give a single enantiomer of the decarboxylation product, (*R*)-acetoin. A gene coding for  $\alpha$ -acetolactate decarboxylase from *Bacillus brevis* (ATCC 11031) was cloned and overexpressed in *B. subtilis*. The enzyme was purified in two steps to homogeneity prior to crystallization. Three different diffraction-quality crystal forms were obtained by the hanging-drop vapour-diffusion method using a number of screening conditions. The best crystal form is suitable for structural studies and was grown from solutions containing 20% PEG 2000 MME, 10 mM cadmium chloride and 0.1 M Tris-HCl pH 7.0. They grew to a maximum dimension of approximately 0.4 mm and belong to the trigonal space group  $P3_1,2,21$ , with unit-cell parameters  $a = 47.0$ ,  $c = 198.9$  Å. A complete data set was collected to 2 Å from a single native crystal using synchrotron radiation.

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## 1. Introduction

Acetolactate decarboxylase [(*S*)-2-hydroxy-2-methyl-3-oxobutanoate carboxy-lyase; EC 4.1.1.5; ADC] catalyses the decarboxylation of (*S*)- $\alpha$ -acetolactate [(*S*)-2-hydroxy-2-methyl-3-oxobutanoate] (1) and (*S*)- $\alpha$ -acetoxybutyrate [(*S*)-2-ethyl-2-hydroxy-3-oxobutanoate] (2) (Fig. 1; Dolin & Gunsalus, 1951; Størmer, 1967; Løken & Størmer, 1970; Hill *et al.*, 1979). (*S*)- $\alpha$ -Acetolactate (1) and (*S*)- $\alpha$ -acetoxybutyrate (2) are the biosynthetic precursors of valine (3) and isoleucine (4), respectively (Fig. 1). The products of decarboxylation of the  $\beta$ -ketocarboxylates (1) and (2) are (*R*)-acetoin (5) and (*R*)-3-hydroxypentan-2-one (6) (Fig. 1), respectively. ADC has found practical application in brewing, where it can be used to speed maturation by catalysing the non-oxidative decarboxylation of  $\alpha$ -acetolactate, thereby avoiding oxidative

decarboxylation to biacetyl, which gives an off-odour to beer (Godtfredsen & Ottesen, 1982; Godtfredsen, Rasmussen, Ottesen, Mathiasen *et al.*, 1984; Godtfredsen, Rasmussen, Ottesen, Rafn *et al.*, 1984).

Although (*S*)- $\alpha$ -acetolactate (1) and (*S*)- $\alpha$ -acetoxybutyrate (2) are the normal substrates of ADC, the enzyme remarkably also catalyses the decarboxylation of the corresponding (*R*)-enantiomers, although at a lower rate. Decarboxylation of both enantiomers of  $\alpha$ -acetolactate leads to a single (*R*)-enantiomer (5) of acetoin (3-hydroxybutan-2-one). Detailed investigation has shown that the enzyme accomplishes this remarkable feat as shown in Fig. 2, by first decarboxylating the (*S*)-enantiomer (1) with overall inversion of configuration at the  $\alpha$ -centre (Crout *et al.*, 1984) and by then catalysing a tertiary ketol rearrangement of the (*R*)-enantiomer (7) with migration of the carboxylate group. The rearrangement proceeds *via* a conformation in which the two oxygen substituents have a *syn* orientation as shown. Because the carboxylate migration proceeds through a *meso* transition state, the configuration at the migration terminus perforce is the opposite of that at the original chiral centre. The rearrangement is degenerate in that the product is still  $\alpha$ -acetolactate but with the (*S*)-configuration (8) (Fig. 2). The tertiary ketol rearrangement thus effects the racemization of  $\alpha$ -acetolactate. The process is reversible in principle, but in practice is driven to completion by the decarboxylation of the (*S*)-enantiomer formed. Thus, eventually all of the racemic acetolactate substrate is decarboxylated to (*R*)-acetoin (5) after the

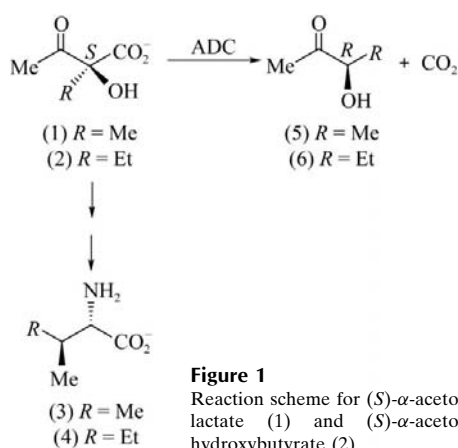


Figure 1

Reaction scheme for (*S*)- $\alpha$ -acetolactate (1) and (*S*)- $\alpha$ -acetoxybutyrate (2).

(*R*)- $\alpha$ -acetolactate (7) has first been epimerized to the (*S*)-enantiomer (8) (Fig. 2).

When  $\alpha$ -acetohydroxybutyrate (2) is the substrate, the rearrangement is no longer degenerate. The (*S*)-enantiomer (2) is decarboxylated to (*R*)-3-hydroxypentan-2-one (6) (Fig. 3). The initial rearrangement of the (*R*)-enantiomer (9) gives the structural isomer (*S*)-2-hydroxy-2-methyl-3-oxopentanoate (10), which then undergoes decarboxylation to (*R*)-2-hydroxypentan-3-one (11) (Fig. 3; Crout & Rathbone, 1988; Crout *et al.*, 1990; Crout, Lee *et al.*, 1991; Crout, McIntyre *et al.*, 1991).

In order to obtain further evidence for the remarkable transformations catalysed by ADC, we have initiated an investigation of the X-ray crystallographic structure of the enzyme.

## 2. Materials and methods

### 2.1. Cloning, expression and purification

The strain used for ADC production, JA222, was a *Bacillus subtilis* multicopy *aldB* derivative of ToC46 (Diderichsen *et al.*, 1990). The multiple copies of *aldB* from *B. brevis* (ATCC 11031) in strain JA222 were made first by integrating one copy of the *aldB* gene into the chromosome downstream of the *dal* gene of ToC46. By intro-

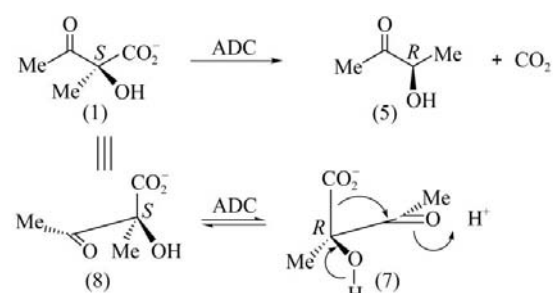
ducing additional copies of *aldB* into the first copy by integration and amplification of plasmid pJA199 using the principle described by Jørgensen *et al.* (2000), the first copy of *aldB* is expressed from the promoter of the maltogenic  $\alpha$ -amylase from *B. stearothermophilus* (Diderichsen & Christiansen, 1988). The additional copies of the *aldB* gene are expressed from the integrated plasmid pJA199 that harbours the *aldB* gene from *B. brevis* expressed from the  $\alpha$ -amylase promoter of *B. licheniformis*, the kanamycin gene (Km) from plasmid pUB110 (McKenzie *et al.*, 1986) and the origin(+) of replication from the temperature-sensitive plasmid pE194 (Horinouchi & Weisblum, 1982).

Terrific Yeast (TY; Diderichsen *et al.*, 1990) was used as liquid media. Luria-Bertani medium (LB; Sambrook *et al.*, 1989) containing 10 mM potassium phosphate pH 7.0, 0.4% (v/w) glucose and 1.5% (v/w) agar, was used as solid media. 250 ml of the supernatant from the fermentation of *B. subtilis* strain JA222 in which ADC was expressed was sterile filtered under pressure using Seitz EKS depth filters purchased from Seitz Schenk Filter System, Germany. The concentration of the filtered supernatant was adjusted to below 4 mM using distilled water and the pH was adjusted to 4.8 by adding dilute acetic acid. A cation-exchange SP-Sepharose Fast Flow (Pharmacia Biotech) column was equilibrated with 50 mM ammonium acetate pH 4.8. The fermentation supernatant was then applied to the column and washed with 50 mM ammonium acetate pH 4.8. ADC has an isoelectric point of around 6 and was bound to the cation exchanger at pH 4.8. Bound protein was eluted using a linear gradient of 50 mM ammonium acetate containing 0–1 M NaCl buffer. All fractions containing ADC were pooled and diluted

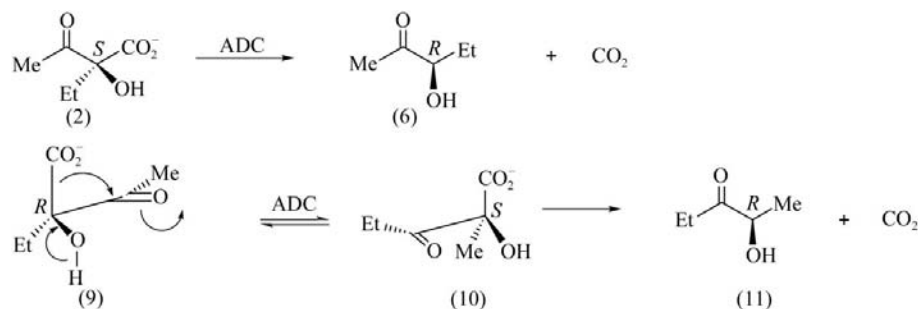
with water to adjust the concentration to below 4 mM and the pH was adjusted to 8.5 with dilute NaOH. In the second step, a Fast Flow Q-Sepharose column was pre-equilibrated with 50 mM Tris-HCl pH 8.5 buffer. ADC was eluted using a linear gradient of 0–1 M NaCl buffer. Fractions containing ADC were then pooled and the pH was adjusted to 7.7 using dilute acetic acid. For identification, the N-terminal amino-acid sequence of the first 18 amino-acid residues was determined. Protein purity was checked by SDS-PAGE and its activity was monitored by the colorimetric method (Crout *et al.*, 1984).

### 2.2. Crystallization

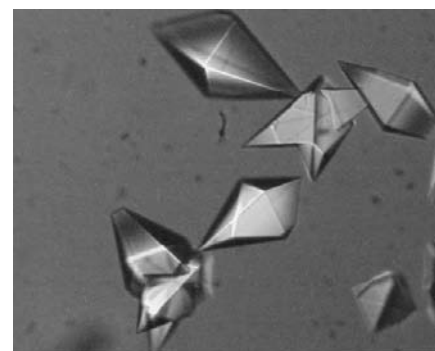
An initial crystallization screen was performed using Hampton Research Crystal Screens 1 and 2 at 291 K with the hanging-drop vapour-diffusion method. 1  $\mu$ l of the protein sample (of concentration  $\sim 10$  mg ml<sup>-1</sup>) and 1  $\mu$ l precipitant was mixed and equilibrated with 0.5 ml precipitant in the well. Crystals appeared in wells 6, 20 and 45 of Crystal Screen 1 after a few days. After refining the initial conditions in a systematic way, bipyramidal crystals (type I; Fig. 4a) were obtained under the following conditions: 10–18% PEG 8000, 0.1 M MES



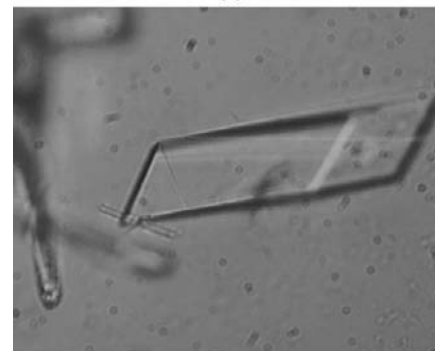
**Figure 2**  
Decarboxylation of both enantiomers of  $\alpha$ -acetolactate leads to a single (*R*)-enantiomer (5) of acetoin (3-hydroxybutan-2-one).



**Figure 3**  
Reaction scheme for  $\alpha$ -acetohydroxybutyrate (2).



(a)



(b)

**Figure 4**  
Photographs of (a) type I and (b) type II ADC crystals. The largest dimension is 0.4 mm for both types.

**Table 1**  
Data-collection and processing statistics.

Values in parentheses correspond to the outer resolution shell.

	Type I	Type II	Type III
Synchrotron source	SRS 14.2	SRS 14.1	SRS 9.6
Wavelength (Å)	0.979	1.488	0.870
Space group	<i>P</i> <sub>4</sub> <sub>2</sub> <sub>2</sub>	<i>P</i> <sub>3</sub> <sub>1</sub> <sub>2</sub>	<i>I</i> <sub>2</sub> <sub>2</sub>
Unit-cell parameters			
<i>a</i> (Å)	61.0	47.0	95.2
<i>b</i> (Å)	61.0	47.0	108.4
<i>c</i> (Å)	185.3	198.9	175.9
Matthews coefficient (Å <sup>3</sup> Da <sup>-1</sup> )	3.0	2.2	2.6
Moecules per AU	1	1	3
Solvent content (%)	58	43	52
Resolution range (Å)	44–2.2	29–2.0	56–2.7
Total observations	99914	141276	106007
Unique reflections	18565	18132	25914
Average <i>I</i> /σ( <i>I</i> )	25.8 (3.0)	34.0 (18.9)	16.2 (3.0)
<i>R</i> <sub>merge</sub>	0.057 (0.395)	0.052 (0.064)	0.090 (0.340)
Completeness	99.2 (100.0)	99.4 (97.5)	99.9 (100.0)

pH 6.0–6.5 and 50–200 mM zinc acetate. Further crystallization trials were performed using other commercial screens (Molecular Dimensions 3D structure screens 1 and 2 and Emerald Biostructures Wizard I and II screens) in conjunction with Hampton Research Additive Screens 1, 2 and 3. Rectangular diffraction-quality crystals (type II; Fig. 4*b*) were obtained on addition of 0.01 *M* cadmium chloride to condition No. 10, Wizard screen I (20% PEG 2000 MME, 0.1 *M* Tris–HCl pH 7.0). This condition with the addition of 3 mM mercury acetate gave a different crystal form (type III).

### 2.3. X-ray diffraction analysis

Single crystals were transferred in a nylon loop to cryoprotectant containing 15% ethylene glycol in the mother liquor and cooled to 100 K for data collection. Initial

experiments were carried out at the ESRF, but all complete data sets were collected at the SRS, Daresbury using ADSC Q4 CCD detectors. All data were indexed, integrated and scaled using the *HKL* suite of programs (Otwinowski & Minor, 1997). Data-processing statistics are given in Table 1. Type I and II forms diffract X-rays beyond 2 Å resolution and contain only one molecule in the crystallographic asymmetric unit and therefore are the prime candidates for obtaining the crystal structure. A sequence-similarity search of ADC against known protein structures identified pyruvate decarboxylase (PDC) from

*Zymomonas mobilis* as having the highest identity. PDC is a much larger three-domain protein and alignment of the first 110 amino-acid residues of ADC with the N-terminal domain of PDC gave 40% sequence similarity. The last 130 residues of ADC are 35% similar to the C-terminal domain of PDC. These low similarity indices correspond to only 5 and 8% identity, respectively. Subsequently, molecular replacement failed to give the correct solution using the PDC domains as search models. A heavy-atom search is in progress in order to solve the structure by multiple isomorphous replacement.

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