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Crystallization and preliminary X-ray analysis of β-alanine synthase from the yeast *Saccharomyces kluyveri*

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In eukaryotes and some bacteria, the third step of reductive pyrimidine catabolism is catalyzed by β-alanine synthase (EC 3.5.1.6). Crystals of the recombinant enzyme from the yeast *Saccharomyces kluyveri* were obtained using sodium citrate as a precipitant. The crystals belong to space group $P2_1$ (unit-cell parameters a = 117.2, b = 77.1, c = 225.5 Å, β = 95.0°) and contain four homodimers per asymmetric unit. Data were collected to 2.7 Å resolution. Introduction of heavy atoms into the crystal lattice induced a different set of unit-cell parameters (a = 61.0, b = 77.9, c = 110.1 Å, β = 97.2°) in the same space group $P2_1$, with only one homodimer per asymmetric unit.

1. Introduction

β-Alanine synthase (βAS; EC 3.5.1.6), also called β-ureidopropionase or *N*-carbamyl-β-alanine amidohydrolase, catalyzes the terminal reaction of the reductive pyrimidine catabolic pathway, in which uracil and thymine are metabolized to β-alanine and β-aminoisobutyrate, respectively. The first and the rate-limiting step in the pathway is the reduction of the pyrimidine ring by dihydropyrimidine dehydrogenase (EC 1.3.1.2); this is followed by hydrolytic ring opening catalyzed by dihydropyrimidinase (EC 3.5.2.2). Finally, βAS is responsible for the irreversible hydrolysis of the intermediate products, *N*-carbamyl-β-alanine and *N*-carbamyl-β-aminoisobutyric acid, which is accompanied by the release of ammonia and carbon dioxide (Wasternack, 1980).

The main function of the pyrimidine catabolic pathway is continuous turnover of pyrimidine bases in the cell, thus maintaining a balanced supply of precursors for nucleic acid synthesis. In addition, this pathway supplies β-alanine. This unusual amino acid is a building block in the biosynthesis of pantothenic acid in bacteria and fungi (Cronan *et al.*, 1982), and of neurologically active dipeptides such as carnosine and anserine in mammals. Owing to its structural analogy to γ-amino-*n*-butyric acid (GABA), it has been suggested that β-alanine itself is also involved in neurotransmission (Sandberg & Jacobson, 1981). In humans, deficiencies in the pyrimidine catabolic pathway, which constitutes the sole source of β-alanine in animal tissues (Traut & Jones, 1996), are associated with severe neurological disorders, seizures and death (van Gennip *et al.*, 1997). Furthermore, this pathway is involved in the efficient degradation of cytotoxic pyrimidine analogues, such as the widely used 5-fluorouracil, which are employed in the treatment of a variety of common tumours, such as colorectal, head/neck and breast cancer (Heggie *et al.*, 1987).

βAS has been purified from calf (Waldmann & Schnackerz, 1989), rat (Tamaki *et al.*, 1987) and mouse (Sanno *et al.*, 1970), and partially purified from maize (Walsh *et al.*, 2001), *Euglena gracilis* (Wasternack *et al.*, 1979) and *Clostridium uracilium* (Cambell, 1960). The βAS genes from rat (Kvalnes-Krick & Traut, 1993), human (Vreken *et al.*, 1999), *Arabidopsis thaliana* (Walsh *et al.*, 2001; Gojković *et al.*, 2001), *Drosophila melanogaster*, *Dictyostelium discoideum* and *Saccharomyces kluyveri* (Gojković *et al.*, 2001) have been cloned and the corresponding enzymes overexpressed as recombinant proteins.

The βASs from rat, maize and *A. thaliana* have been further characterized. All share a subunit molecular mass of 42–44 kDa (Tamaki *et al.*, 1987; Walsh *et al.*, 2001). Rat βAS and *A. thaliana* βAS exist as different oligomeric species depending either on the presence of allosteric effectors (Matthews & Traut, 1987) or on pH (Walsh *et al.*, 2001). The native βAS from maize appears to be present as a highly oligomerized enzyme (at least a decamer) also in the absence of substrate or product (Walsh *et al.*, 2001). Metal analyses have been performed for the rat and maize enzymes and both have been shown to contain Zn^{2+} ions (Kvalnes-Krick & Traut, 1993; Walsh *et al.*, 2001).

Multiple alignment and phylogenetic analysis of the available sequences of βAS and related carbamyl amidohydrolases have revealed that the enzymes can be grouped into three subfamilies (Gojković *et al.*, 2001). While the analysed mammalian and most other

eukaryotic βAS form one subfamily (with pairwise sequence identities of 55–85%), the enzyme from the yeast *S. kluyveri* clusters together with bacterial *N*-carbamyl-L-amino acid amidohydrolases (30–41% sequence identity). A group of proteins with average identities of not more than 20%, including aminoacylases and peptidases of the M20/M25/M40 family (Rawlings & Barrett, 1995), is a distant relative of this subfamily. The third subfamily consists of *N*-carbamyl-D-amino acid amidohydrolases. Recently, the three-dimensional structures of two enzymes from this subgroup have been reported (Nakai *et al.*, 2000; Wang *et al.*, 2001).

In this paper, we report the crystallization and preliminary X-ray diffraction data analyses of the first βAS, *S. kluyveri* βAS, which belongs to the second subfamily of carbamyl amidohydrolases.

2. Results

2.1. Crystallization

S. kluyveri βAS was expressed with a C-terminal tag of eight histidines (plasmid P491) and purified on an Ni^{2+}–NTA column (Qiagen) as described previously (Gojković *et al.*, 2001). Incorporation of selenomethionine was accomplished in the following way. P491 was introduced into the *Escherichia coli* B843(DE3) strain, which is a methionine auxotroph (Studs & Fox, 1999). 2.5 ml of the overnight culture was inoculated into 500 ml of MOPS medium (Neidhardt *et al.*, 1974) with 45.6 mg l^{-1} L-methionine and 100 mg l^{-1} ampicillin and the cells were grown to an OD_{600} of 0.25. The cells were then washed and centrifuged and the pellet was subsequently inoculated into 500 ml of fresh MOPS medium with 60 mg l^{-1} seleno-L-methionine and 100 mg l^{-1} ampicillin and grown to an OD_{600} of 0.50. Expression was induced by the addition of anhydrotetracycline as described in Gojković *et al.* (2001). The final protein product does not contain any non-native residues other than the histidine tag or the incorporated selenomethionine.

Sparse-matrix crystallization screens (Hampton Research) were set up at 277 and 293 K using the hanging-drop vapour-diffusion method, but did not result in any protein crystals. Follow-up screens based on the most frequently used precipitants such as polyethylene glycol and ammonium sulfate yielded the same result. Organic salts were explored next and after 14 d of equilibration spindle-shaped bundles of thin needles were obtained at room temperature with a reservoir solution comprising 0.1 *M* sodium citrate pH 5.5, 0.8 *M* trisodium citrate and an initial concentration of 2.5 mg ml^{-1} βAS in the drop. This condition was further optimized by variation of precipitant and protein concentration and buffer pH, and by the utilization of additives. The best crystals were obtained using vapour diffusion against either 0.88 *M* trisodium citrate, 0.1 *M* sodium citrate pH 6.0, 5%(*v*/*v*) dioxane or 1.0 *M* trisodium citrate, 0.1 *M* MES pH 6.5, 5%(*v*/*v*) dioxane. The drops consisted of 3 µl protein solution (4.0–4.5 mg ml^{-1} in 50 m*M* Tris–HCl pH 7.5, 1 m*M* DTT, 100 m*M* NaCl) and 3 µl reservoir solution and were equilibrated against a 1 ml reservoir at room temperature. Crystals appeared within 5 d and reached maximum dimensions of 0.25 × 0.1 × 0.5 mm (Fig. 1). Crystals of selenomethionine-substituted protein grew under the same conditions to comparable size, but required a further 2 d for appearance.

2.2. Data collection and analysis

All data processing, scaling and truncation was performed using the programs *MOSFLM*, *SCALA* and *TRUNCATE* from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994).

Native data were collected as 0.45° oscillations at 100 K and a wavelength of 0.933 Å on beamline ID14-1 at the ESRF (Grenoble, France) using an ADSC Q4R CCD detector. No cryoprotectant was required. Crystals of native βAS belong to the monoclinic space group $P2_1$, with unit-cell parameters $a = 117.2$, $b = 77.1$, $c = 225.5$ Å, $\beta = 95.0°$, and diffracted to a maximum resolution of 2.4 Å. βAS crystals are sensitive to radiation damage, as judged by the fast decay of the high-resolution reflections. As a consequence, only data to 2.7 Å resolution could be used after collection of a complete set. Data statistics are given in Table 1. The value of the Matthews coefficient (Matthews, 1968) is 2.56 $Å^3$ Da^{-1} for eight molecules in the asymmetric unit, which corresponds to a solvent content of 51.6%.

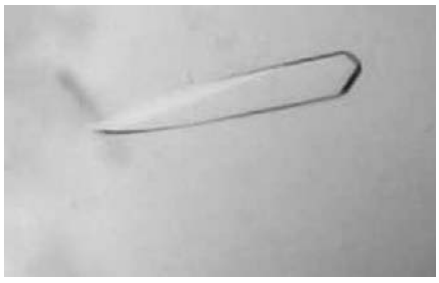

Figure 1
A crystal of recombinant *S. kluyveri* βAS as grown by the hanging-drop method. The largest dimension is ~0.2 mm.

Table 1
Diffraction, data collection and reduction statistics.

Values in parentheses are for the highest resolution shell (2.85–2.70 Å).

Wavelength (Å)	0.933
Space group	$P2_1$
Unit-cell parameters (Å, °)	$a = 117.2$, $b = 77.1$, $c = 225.5$, $\beta = 95.0$
Resolution limits (Å)	25.0–2.7
No. of reflections	366678
No. of unique reflections	106190
R_{sym} (%)	8.3 (26.1)
Mean $I/\sigma(I)$	14.8 (3.7)
Completeness (%)	95.9 (82.2)
Multiplicity	3.5 (2.6)

Attempts to solve the structure of βAS by molecular replacement using the structures of carboxypeptidase G2 (Rowsell *et al.*, 1997), a member of the M20/M25/M40 family of peptidases, and *N*-carbamyl-D-amino acid amidohydrolase (Nakai *et al.*, 2000) from the third subfamily of carbamyl amidohydrolases remained unsuccessful.

In order to solve the structure by isomorphous replacement methods, we soaked crystals of native βAS in heavy-metal solutions and data sets were collected for several candidates. However, we observed that the soaked crystals exhibited two distinct sets of unit-cell parameters in the same space group $P2_1$, apparently depending on the type and concentration of the salt and the soaking time. While crystals soaked with heavy-metal compounds such as tantalum bromide and ytterbium chloride exhibit the same unit-cell parameters as crystals of native βAS, we obtained parameters of $a = 61.0$, $b = 77.9$, $c = 110.1$ Å, $\beta = 97.2°$ with, for example, crystals soaked in holmium chloride and uranium nitrate. For other soaks, we observed a transformation from the large to the small unit cell during X-ray exposure, making the collected data useless for phase determination. It is noteworthy that the dimensions of the smaller unit cell are related to those of the larger unit cell by a bisection of the dimensions *a* and *c*, resulting in a fourfold smaller asymmetric unit containing only two polypeptide chains. Taking into account the relation between the two sets of unit-cell parameters and the observation that βAS from *S. kluyveri* exists as a dimer in solution (unpublished results), we expected the positions of the four dimers in the asymmetric unit to be related either by (almost pure) translational vectors or by a combination of crystallographic symmetry and translation. This is consistent with the appearance of three translation peaks in the

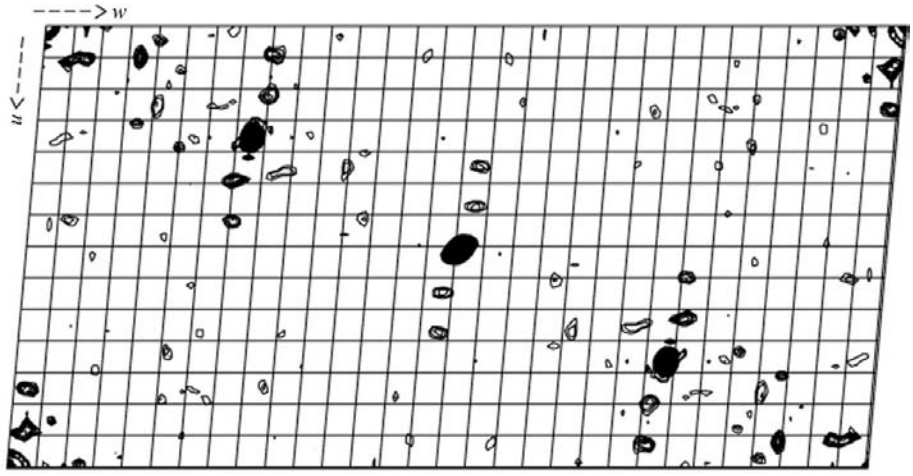

Figure 2
Section $v = 0$ of the native Patterson map calculated at 15.0–4.0 Å for *S. kluyveri* βAS.

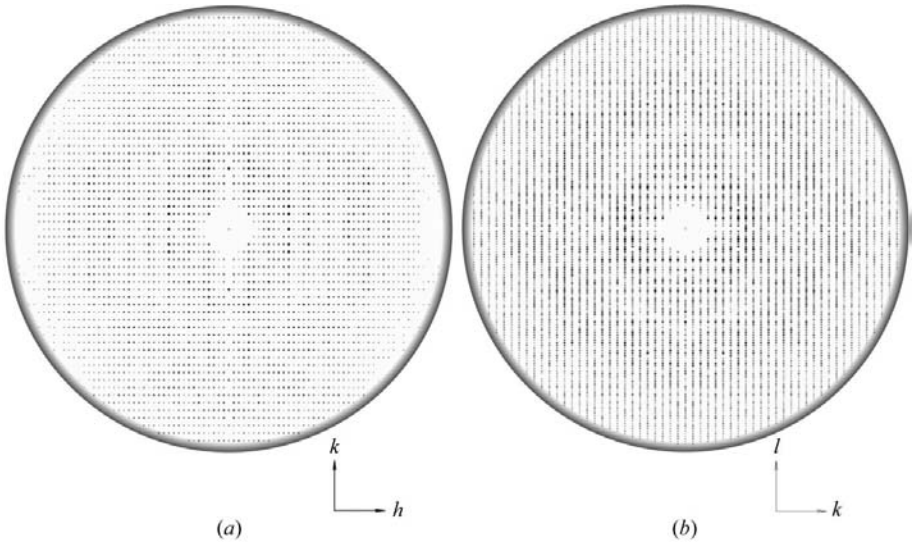

Figure 3
Pseudo-precession images of (*a*) the *hk*0 layer and (*b*) the 0*kl* layer calculated with the program *PATTERN* (Lu, 1999).

$v = 0$ section of the native Patterson map at the positions (0.251, 0, 0.249), (0.500, 0, 0.500) and (0.749, 0, 0.751) (Fig. 2). A special mode of molecular packing within the asymmetric unit is also evident from the pseudo-precession images of the *hk*0 and 0*kl* layers, calculated from the X-ray data with the program *PATTERN* (Lu, 1999), in which every fourth reflection in either the *h* and *l* direction appears to be stronger than the others (Fig. 3).

Since the determination of the structure by isomorphous replacement methods was not straightforward owing to the observed changes in the unit-cell parameters, we decided to use the MAD phasing technique instead. Data have been collected from crystals of selenomethionine-substituted βAS, which exclusively exhibit the smaller unit-cell with parameters $a = 60.4$, $b = 77.6$, $c = 110.6$ Å, $\beta = 95.6^\circ$, and full structure analysis is currently under way.

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