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Crystallization of *Arthrobacter* sp. strain 1C *N*-(1-D-carboxyethyl)-L-norvaline dehydrogenase and its complex with NAD^+

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Abstract

The novel NAD^+-linked opine dehydrogenase from a soil isolate *Arthrobacter* sp. strain 1C belongs to an enzyme superfamily whose members exhibit quite diverse substrate specificites. Crystals of this opine dehydrogenase, obtained in the presence or absence of co-factor and substrates, have been shown to diffract to beyond 1.8 Å resolution. X-ray precession photographs have established that the crystals belong to space group $P2_12_12$, with cell parameters a = 104.9, b = 80.0, c = 45.5 Å and a single subunit in the asymmetric unit. The elucidation of the three-dimensional structure of this enzyme will provide a structural framework for this novel class of dehydrogenases to enable a comparison to be made with other enzyme families and also as the basis for mutagenesis experiments directed towards the production of natural and synthetic opine-type compounds containing two chiral centres.

1. Introduction

Opines are the products of the NAD(P)H-dependent reductive condensation between an α-keto acid and the α- or ω-NH_2 group of an amino acid in a reaction catalyzed by a family of enzymes generically referred to as opine dehydrogenases. The products of these reactions have two asymmetric centres and, in Nature, opines may exhibit either (L,L) or (D,L) stereochemistry. Compounds belonging to this class have been isolated from eukaryotic cells, plant tumours, bacteria and muscle tissue of marine invertebrates (reviewed in Thompson & Donkersloot, 1992).

Amino-acid sequences are currently available for five opine dehydrogenases. Three of these enzymes are responsible for the synthesis of opines with (D,L) stereochemistry (nopaline, lysopine and opine dehydrogenase) (Bevan *et al.*, 1983; Barker *et al.*, 1983; Asano *et al.*, 1989) whilst the remaining two catalyze the synthesis of opines with (L,L) stereochemistry (saccharopine dehydrogenase and carboxyethyl ornithine synthase) (Xuan *et al.*, 1990; Donkersloot & Thompson, 1995). Analysis of the amino-acid sequences and biochemical studies have established that the enzymes which catalyse the (D,L) and (L,L) chemistry belong to two apparently distinct superfamilies with differential specificity for the keto acid and amino-acid partners. The enzymes within one family display differential substrate specificity but share sequence similarities in the range 20–30% identities, whereas there is no detectable sequence similarity between the families. This implies that the three-dimensional structure within a family but not between families will be similar.

The lysopine and nopaline families of opines found in crown gall tumours are the most extensively studied of the naturally occurring opines (Thompson & Miller, 1991). Such tumours are widespread on many dicotyledenous plants (Nester *et al.*, 1981) and these hyperplasias (Nester & Kosuge, 1981) were first described by Aristotle (referenced in Drummond, 1979). However, it was not until 1907 that the causal relationship between infection of wounded plants by agrobacteria and tumour etiology was established (Smith & Townsend, 1907). It is now known that the enzymes catalysing the synthesis of opines are found in transformed plant cells. However, the structural genes for these enzymes are encoded on large plasmids resident in virulent strains of agrobacterium (DeGreve *et al.*, 1981). These tumour-inducing plasmids are required for crown gall induction, and tumorigenesis involves the excision of a segment of the plasmid DNA on which the opine dehydrogenase gene is located. Following integration of this DNA into the plant genome (Chilton *et al.*, 1977), the plant cell machinery is hijacked to divert resources to the synthesis of opines which are transported to the gall to allow growth of the tumour. Further studies have shown that opines play a major role at several stages of the intricate prokaryote–eukaryote inter-relationship between virulent strains of *A. tumefaciens* and plants including increasing the transcription of the Ti plasmid-encoded virulence (*vir*) genes (Veluthambi *et al.*, 1989).

A major area of interest in this enzyme superfamily lies in the field of enzyme-based chiral synthesis. Opine-type secondary amine dicarboxylic acids are useful chiral intermediates for a number of important pharmaceuticals, for example the antihypertensive agents Enalaprilat and Lysinopril. Given that such compounds contain multiple chiral centres the synthesis of such compounds by enzyme-based technology, rather than classical chemistry, has enormous potential. Thus, the rational development of opine dehydrogenases with novel specificities through mutagenesis, could have important applications in areas of drug production and discovery. The rational modification of the specificity of these enzymes would require the determination of the molecular structure of representative members of the opine dehydrogenase family and as yet there are no structures for any member of either of these families and the molecular basis of the substrate specificity and catalytic activity are unknown.

The opine dehydrogenase from the soil isolate *Arthrobacter* sp. strain 1C *N*-(1-D-carboxyethyl)-L-norvaline dehydrogenase (CENDH) belongs to the (D,L) family and exists as a homodimer with a subunit molecular weight of about 36 000 (Asano *et al.*, 1989). In the oxidative deamination reaction, the enzyme is active towards opines, such as *N*-[1-D-(carboxyl)-ethyl]-L-methionine (methiopine) and *N*-[1-D-(carboxyl)ethyl]-L-phenylalanine. In the reductive secondary amine forming reactions with NADH as cofactor, the enzyme utilizes hydrophobic L-amino acids, such as L-methionine, L-isoleucine,

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L-valine, L-phenylalanine and L-leucine, as amino donors and α-keto acids, such as pyruvate and oxaloacetate, glyoxylate and α-ketobutyrate, as amino acceptors (Dairi & Asano, 1995).

In this paper we report the crystallization of the opine dehydrogenase from *Arthrobacter* sp. strain 1C together with the preliminary results towards the determination of the X-ray crystal structure of this enzyme.

2. Experimental

Escherichia coli cells harbouring the plasmid containing the opine dehydrogenase gene (JM109/pODH1) were aerobically cultivated at 310 K for 10 h in a LB medium containing 1% of tryptone, 0.5% of yeast extract, and 1% of NaCl, pH 7.5; thereafter, 1 m*M* of IPTG (final concentration) was added to the culture to induce expression of the *odh* gene, and incubation was continued for a further 7 h. Cells (about 565 g wet weight) from 120 l (240 batches of 500 ml medium in 2 l flasks) of culture were suspended in 0.1 *M* buffer. The enzyme was purified from the cells of the overexpressing strain as described previously by Asano *et al.* (1989) except for the insertion of an additional purification step between the hydrophobic chromatography and gel-filtration steps. This involved the separation of the enzyme on a Q-Sepharose 35/100 column equilibrated with 20 m*M* potassium phosphate buffer, pH 7.0, containing 0.1 m*M* EDTA and 5 m*M* 2-mercaptoethanol, eluting the enzyme with a linear gradient of NaCl (0–0.2 *M*) in 20 m*M* buffer. The enzyme solution was then dialysed and concentrated by ultrafiltration before being applied to a Superdex 200TM 35/600 column equilibrated with the buffer (0.05 *M*) containing 0.1 *M* NaCl. The active fractions were collected, concentrated by ultrafiltration and stored at 253 K in 50% glycerol.

For crystallization, samples of the stored protein solution were dialysed against 0.1 *M* potassium phosphate buffer, pH 6.0. The concentration of the enzyme was adjusted to give an optical density at 280 nm of approximately 10 (corresponding to a concentration of around 10 mg ml^{-1}) using an Amicon centricon 30 microconcentrator by centrifugation at 4000*g* in a Beckman J2-21 refrigerated centrifuge. Crystallization trials were carried out using the hanging-drop method of vapour diffusion by mixing 5 µl of the protein solution with 5 µl of the precipitant and equilibrating the drops over the precipitant at 290 K. Trials with ammonium sulfate in a concentration range 34–40% saturation in 0.1 *M* potassium phosphate buffer pH 6.0 were carried out in the presence and absence of 10 m*M* NAD^+, or 10 m*M* NAD^+ and 10 m*M* phenylalanine. Under all conditions single crystals with rhomboid morphology and maximum dimensions of 0.5 × 0.3 × 0.3 mm were obtained after two weeks over an ammonium sulfate concentration range of 36–37% for the trials on the apoenzyme; 38–39% in the presence of 10 m*M* NAD^+ and 36–40% in the presence of 10 m*M* NAD^+ and 10 m*M* phenylalanine.

These crystals were confirmed to be those of *Arthrobacter* CENDH by washing a single crystal, which had been subject to X-ray analysis, with 45% saturated ammonium sulfate in 0.1 *M* potassium phosphate buffer pH 6.0 followed briefly with water and then determining the amino-acid sequence for the first ten residues on an Applied Biosystems 476A sequencer.

3. Results and discussion

X-ray precession photographs of the CENDH crystals grown in the presence of 10 m*M* NAD^+ showed that they belong to the orthorhombic system, point group 222 with cell dimensions a = 104.9, b = 80.0, c = 45.5 Å and a cell volume of 3.8×10^5 $Å^3$. Analysis of the diffraction data showed that axial reflections along the h and k axes were present only for even ordered reflections, with all other reflections present identifying the space group as $P2_12_12$. Gel-filtration studies have shown that CENDH is dimeric with a subunit molecular weight of 36 000 (Asano *et al.*, 1989) and thus if the asymmetric unit contains a single subunit then the V_m is 2.65 $Å^3$ Da^{-1} which is well within the range given by Matthews (1977). The V_m value for a dimer in the asymmetric unit falls outside the range. Consequently, the CENDH dimer must be constructed using the crystallographic twofold axis along c.

Preliminary images recorded on station PX9.5 at the CLRC Daresbury Laboratory Synchrotron Radiation Source (SRS), indicate that the crystals diffract to beyond 1.8 Å (Fig. 1). A total of 62.5° of data have been collected from two ODH crystals grown in the presence of 10 m*M* NAD^+ at the SRS to a resolution of 1.8 Å using the rotation method of data collection and rotations of 1.3° per frame. Observations were recorded on a large MAR Research image plate at an X-ray wavelength of 0.90 Å. 66 518 measurements were made of 30 854 independent reflections and the data set processed using the *MOSFLM* (Leslie, 1992) and *CCP*4 (Collaborative Computational Project, Number 4, 1994) software packages. The crytals showed no appreciable radiation damage during data collection. The data were merged to an R factor of 4.0%, $I/\sigma(I)$ of 8.37, multiplicity of 2.3 and 89% completeness of the data from 16 to 1.8 Å resolution. The highest resolution shell (1.85–1.80 Å) gave an R factor of 15.2%, $I/\sigma(I)$ of 5.0, multiplicity of 2.4 and 78% completeness in the range.

An attempt to solve the structure by multiple isomorphous replacement is now under way and the results of this analysis will play a vital role in developing a full understanding of the structure–function relationships in this enzyme.

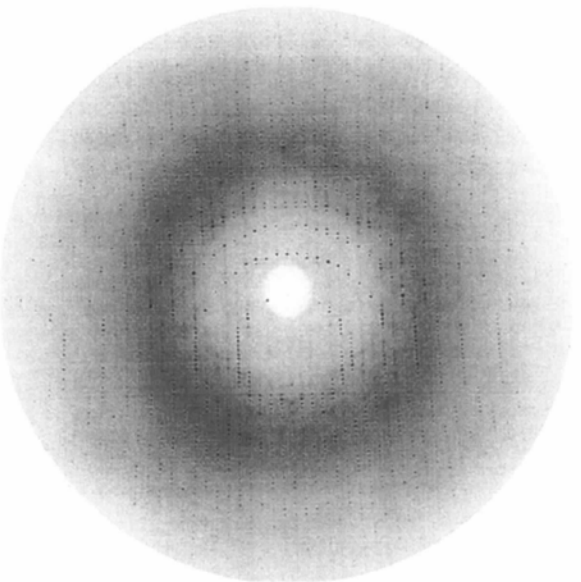

Fig. 1. A 1.3° oscillation image of the *Arthrobacter* sp. strain 1C opine dehydrogenase crystals grown in the presence of NAD^+ using the MAR image plate of station PX9.5 at the SRS. The resolution at the edge of the image is 1.8 Å.

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