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Crystallization and X-ray diffraction analysis of ornithine cyclodeaminase from *Pseudomonas putida*

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Ornithine cyclodeaminase (OCD) is a member of the μ-crystallin protein family, the biological activity of which is the conversion of L-ornithine to L-proline and ammonia. In order to elucidate the functional groups of this enzyme that are involved in catalysis, the crystallization of OCD from *Pseudomonas putida* was undertaken. Using microbatch-under-oil screening at the high-throughput crystallization laboratory (HTC) at the Hauptman–Woodward Medical Research Institute Inc. (HWI Buffalo, NY, USA), numerous crystallization conditions were rapidly identified. Several conditions could be reproduced on a larger scale as vapor-diffusion experiments in-house. The best diffraction-quality crystals were obtained from solutions of 40%(v/v) 2-methyl-2,4-pentanediol buffered at pH 6.0 with 0.1 *M* MES and diffracted X-rays to 1.68 Å resolution. Crystals belonged to space group $P2_12_12_1$, with unit-cell parameters a = 70.0, b = 78.3, c = 119.4 Å. The V_M was 2.1 Å^3 Da^{-1}, corresponding to 42% solvent, which is consistent with two 38.5 kDa molecules per asymmetric unit. The structure determination is under way using experimental phasing methods.

1. Introduction

Ammonia is an important nitrogen source for most bacteria. Ornithine cyclodeaminase is a bacterial enzyme that converts L-ornithine to L-proline and ammonia *via* a pathway (Fig. 1) extant in several bacterial strains including *Pseudomonas*, *Agrobacterium tumefaciens*, *Rhizobium* (Soto *et al.*, 1994), *Clostridium* (Muth & Costilow, 1974) and *Brucella abortus* (Kim & Mayfield, 1997). The reaction requires NAD$^+$, which is believed to function catalytically rather than being irreversibly converted to NADH. No other cofactors or metals are known to be required for catalytic activity.

Sequences homologous to OCD have been identified in a variety of bacterial and archaeal genomes, indicating the importance of this enzyme in growth and survival. In nature, the OCD gene has been found within tumor-inducing plasmids of *Agrobacterium*, which infects plants to form crown-gall tumors. In these pathogens it is believed that OCD is involved in the biosynthesis of opines, which serve as a nutritional source for the bacteria encased within the crown gall (Sans *et al.*, 1987). OCD and its homologs are also of interest because they are related to members of the crystallin protein family. This family also includes μ-crystallin, the major component of the eye lens in several marsupials (Kim *et al.*, 1992). The precise function of these proteins remains elusive, but they may have a regulatory function in the use of amino acids as neurotransmitters (Barnstable, 1993). Recently, Smith *et al.* (2003) reported that a gene from *Archaeoglobus fulgidus*, annotated as an OCD, was in fact a novel alanine dehydrogenase that belonged to the μ-crystallin family. These results suggested that OCDs and related proteins may comprise a novel structural class of NAD$^+$-dependent enzymes.

To investigate the mechanism of OCD and provide an insight into the fold of the μ-crystallin family, we undertook a structural investigation of a 350-amino-acid variant of OCD from the Gram-negative soil bacterium *Pseudomonas putida*. This report describes the expression, purification, crystallization and

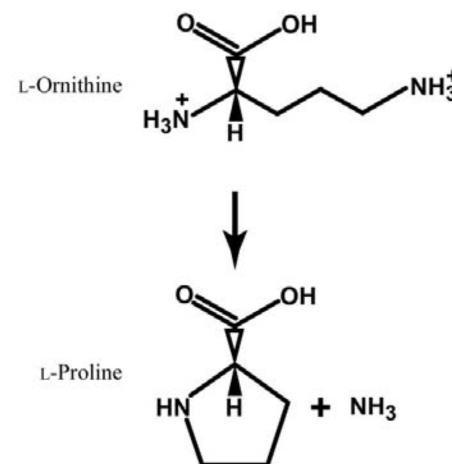

Figure 1
The enzymatic conversion of L-ornithine to L-proline and ammonia by the bacterial enzyme ornithine cyclodeaminase.

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preliminary X-ray diffraction analysis of crystals identified using high-throughput screening methods.

2. Materials and methods

2.1. Cloning

The OCD gene was cloned by PCR from *P. putida* genomic DNA (American Type Culture Collection) using Pfu DNA polymerase (Stratagene). Primers (UW-Madison Biotechnology Center) were designed to incorporate *Nde*I and *Xho*I restriction sites at the start and stop codons, respectively, and to change the stop codon from TGA to TAA. The 1065 bp PCR product was ligated to the topoisomerase-I pCR Blunt II TOPO vector (Invitrogen). The resulting plasmid was digested with *Nde*I and *Xho*I and then subcloned into the pET-21a(+) expression vector (Novagen) digested with *Nde*I/*Xho*I and treated with calf intestine alkaline phosphatase. The pET-21a-OCD plasmid was transformed into *Escherichia coli* XL-2-Blue MRF′ supercompetent cells (Stratagene). After verifying the correct sequence, the plasmid was transformed into *E. coli* Rosetta (DE3) (Novagen) for expression of the OCD protein.

2.2. Protein expression and purification

A 25 ml culture of LB broth supplemented with ampicillin ($100\ \mu g\ ml^{-1}$) was inoculated with a single colony of *E. coli* Rosetta (DE3) carrying pET-21a-OCD and grown overnight at 310 K with vigorous shaking. The overnight culture was used to inoculate fresh LB broth supplemented with ampicillin ($100\ \mu g\ ml^{-1}$) and grown to an OD_{600} of 0.6 at 310 K. Cultures were induced with 0.5 m*M* IPTG and additional ampicillin ($50\ \mu g\ ml^{-1}$) was added to the culture to promote selection of the expression plasmid. Cells were harvested after 3–4 h induction and stored at 193 K.

For protein purification, the cells were thawed, resuspended in three volumes of buffer *A* (20 m*M* NH_2PO_4 pH 8.0) and disrupted by sonication for 30 s. Phenylmethylsulfonyl fluoride (0.5 m*M*) was added to the cell suspension, which was subjected to further sonication. After sonication, 0.2%(*w*/*w*) protamine sulfate was added and the resulting mixture was stirred for 10 min on ice. The homogenate was clarified by centrifugation at 42 000*g* for 30 min at 277 K. The supernatant was loaded onto a 2.5 × 100 cm Q-Sepharose column (Amersham Biosciences) equilibrated with buffer *A*. Protein was eluted using a 0.40 l linear gradient of NaCl (0–1 *M* in buffer *A*). OCD eluted at a NaCl concentration of approximately 0.33 *M*.

The fractions containing OCD were pooled and brought to 1.5 *M* in $(NH_4)_2SO_4$ and stirred for 60 min on ice. After centrifugation at 42 000*g* for 30 min, the supernatant was loaded onto a 2.5 × 100 cm phenyl-Sepharose column (Amersham Biosciences) equilibrated in buffer *B* comprising 20 m*M* NaH_2PO_4 pH 8.0 with 1 *M* $(NH_4)_2SO_4$. Protein was eluted with an initial 0.20 l linear gradient from 100% buffer *B* to 100% buffer *A*, followed by a 0.15 l wash with 100% buffer *A*. Protein eluted during the no-salt wash. The fractions containing OCD were pooled and concentrated using a Centriprep concentrator containing a YM-30 (molecular-weight cutoff 30 kDa) membrane (Millipore). For crystallization trials, the protein was concentrated to $18.2\ mg\ ml^{-1}$ and buffer-exchanged into 10 m*M* HEPES pH 7.5 containing 0.10 *M* NaCl using a Centriprep concentrator. The purity was judged to be >95% as assessed by SDS–PAGE. Protein concentrations were estimated by the method of Waddell (1956). The protein yield was ~0.06 g per litre of cells.

2.3. Crystallization

A screen for initial protein crystallization conditions was conducted at the High Throughput Crystallization Laboratory located at the Hauptman–Woodward Medical Research Institute (Buffalo, NY, USA). Protein was express-shipped in liquid form packed in ice. The initial screening was performed using the Apogen Discoveries/Robbins Scientific Tango liquid-handling system, which enabled a search of 1536 solutions at 277 and 293 K (Luft *et al.*, 2003). The screens were carried out by the microbatch-under-oil method using 0.20 µl protein and 0.20 µl precipitating agent. The screening results were monitored by manual inspection of digital images, retrieved by FTP for home viewing, using the program *MacroScope* (Luft *et al.*, 2003).

2.4. Crystal optimization

Crystallization conditions identified through use of the HTS Laboratory were reproduced in-house. The conditions assessed included those available from commercial screens including Crystal Screen HT (Hampton Research), the PEG/Ion Screen (Hampton Research) and the MPD (2-methyl-2,4-pentanediol) Grid Screen (Hampton Research). Crystals reproduced in-house led to a series of fine screens conducted to optimize crystal growth. This work required chemical stocks of MPD (Fluka), MES (2-morpholinoethanesulfonic acid, Fluka) and HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, Merck). The respective buffers were titrated to pH 6.0 or 7.0 using NaOH. Stock solutions were prepared using Nanopure water (Barnstead). All solutions were passed through a 0.2 µm filter. In-house crystallization trials were conducted using the hanging-drop vapor-diffusion method at 277 and 293 K. The protein was microcentrifuged (18 000*g*) prior to use. The PEG/Ion and MPD Grid screens were conducted using the Crystal Tool (Nextal Biotech), in which 2 µl precipitating agent was removed from a 1 ml volume of the reservoir and added to 2 µl protein solution without mixing. Crystal Screen HT utilized a 96-well sitting-drop format in which 100 µl precipitating agent was dispensed by an analog multi-channel pipette from the deep well block into the reservoir of a Corning crystallography plate (Hampton Research). 2 µl well solution was added to 2 µl protein solution and the plate was sealed with clear tape. Vapor-diffusion experiments were equilibrated for 3 d and then inspected by use of an MZ7.5 dissecting microscope (Leica) equipped with a polarizing filter and capable of 80× magnification.

The substrate L-ornithine (Sigma) or product L-proline (Sigma) was also co-crystallized with OCD under the established conditions. The protein was pre-mixed with 2–8 m*M* concentrations of these respective amino acids and drops were equilibrated for 3 d prior to inspection.

2.5. X-ray diffraction

Single crystals were cryoprotected by serial transfer into synthetic mother-liquor solutions comprising the crystallization medium with successively higher amounts of MPD (40 and 50%). Crystals were captured by surface tension in 20 µm rayon loops (Hampton Research) and flash-cooled to 100 K by exposure to a stream of cold nitrogen gas (X-stream, Rigaku/MSC). The crystal was visualized by a 450× CCD camera (Rigaku/MSC) connected to a color video display. X-ray diffraction data were recorded on an R-AXIS IV image-plate detector equipped with a vertical φ-rotation axis at a crystal-to-detector distance of 150 mm. X-rays were generated by an RU-H2R rotating-anode generator (Rigaku/MSC) operated at 4.5 kW and equipped with a 0.3 mm focal cup. X-rays were filtered and focused by use of confocal 'Blue' optics (Osmic) and collimated through a 0.3 mm

Table 1
X-ray diffraction data and intensity statistics.

Values in parentheses are for the highest resolution shell.

Space group	$P2_12_12_1$
Unit-cell parameters	
a (Å)	70.0
b (Å)	78.3
c (Å)	119.4
$\alpha = \beta = \gamma$ (°)	90
Resolution range (Å)	26.28–1.80 (1.86–1.80)
Unit-cell volume (Å^3)	654032
Solvent content (%)	42
Measured reflections	236931
Unique reflections	63405
Average redundancy	3.92 (1.9)
Data completeness	95.4 (72.8)
$R_{\rm sym}$† (%)	0.074 (0.32)
$I/\sigma(I)$	11.0 (2.7)

† $R_{\rm sym} = [\sum |I(h)_j - \langle I(h)\rangle| / \sum I(h)_j] \times 100$, where $I(h)_j$ is the jth observation of the intensity of the reflection h and $\langle I(h)\rangle$ is the mean intensity of the reflection h from multiple measurements.

front-end pinhole. A data-collection strategy was calculated using the *CrystalClear* software package (Pflugrath, 1999). Diffraction data were recorded in 225 images of 0.5° oscillation per 20 min exposure. Data reduction and scaling were conducted using the *CrystalClear* software. Intensity statistics are reported in Table 1.

3. Results and discussion

3.1. Crystallization trials by automated methods

Use of the high-throughput protein crystallization laboratory (HWI Buffalo, NY, USA) enabled a rapid and extensive screen of crystallization parameters for the OCD protein from *P. putida*. Approximately 14.5 mg of pure protein was expended in this effort to examine 3072 conditions. However, the results indicated that the amount of material could have been reduced by half because most crystallization hits observed at 293 K also produced crystals at 277 K, albeit on a slower timescale. In addition, there were numerous conditions under which crystals appeared at 293 K that did not produce detectable crystals at 277 K. Hence, screening at two temperatures simultaneously should be reserved for cases where protein is abundant but time is not.

For our colleagues who resist employing an HTC laboratory, we would argue that such a facility is much more efficient compared with either conventional methods or the increasingly popular high-throughput methods designed for use in-house. Assuming *a posteriori* that we limited our crystal search to the 2 × 168 conditions represented by the three commercial screens that produced the best OCD crystals, we would still have expended nearly 6.2 mg of protein for trials at 277 and 293 K. This is because the smallest precisely and accurately delivered pipette volume in home trials was 1 µl, which is fivefold greater than that achieved by the HTC robot. Furthermore, the robot takes approximately 8 min to load a 1536-well plate, whereas the average individual in our laboratory required 10–12 min per 24-well plate or 96-well HTC plate. Therefore, in cases where a protein has not been crystallized, the most efficient screening method in terms of minimizing protein and time consumption appears to be the use of an HTC laboratory (Luft *et al.*, 2003).

3.2. Crystallization results

Most crystals from the HTC Laboratory appeared in approximately 2–3 d at 293 K (Fig. 2), but required >6 d at 273 K. Promising lead precipitating agents included polyethylene glycol (PEG) 6000, jeffamine, dioxane, MPD and hexanediol buffered with either 0.1 *M* MES at pH 6.5, 0.1 *M* HEPES pH 7.0 or 7.5, 0.1 *M* Tris pH 8.0 or 8.5 or 0.1 *M* bicine pH 9.0. In most instances, high concentrations of precipitating agents were present when crystals were formed. In contrast, salt was not an essential component in most conditions. A large number of the best single crystals (using criteria of size, thickness and singularity) were observed in conditions derived from three commercially available kits employed by the HTC Laboratory. Under many of the kit conditions showers of crystals were visible, but some single crystals were also visible in conditions comprising 0.1 *M* HEPES pH 7.5, 70%(*v*/*v*) MPD, 35%(*v*/*v*) dioxane and 0.1 *M* MES pH 6.5, 30%(*w*/*v*) PEG 6000.

Upon identifying the lead crystallization conditions, attempts were made to reproduce the results in-house by use of the three commercial screens. The most reproducible conditions were observed with the MPD Grid Screen, which consistently gave single crystals. In order to prevent overnucleation, the MPD screen was refined by evaluating different concentrations of MPD (30–60% in 5% increments) *versus* different pH values of NaMES buffer (pH 5.5–6.5 in 0.5 unit increments). The refinement efforts demonstrated that crystals could be reproduced at home on a 4 µl scale (*i.e.* 10× larger than the HTC Laboratory), resulting in X-ray diffraction-quality crystals. Rod-shaped crystals were visible after 3 d and grew from solutions of 0.1 *M* MES pH 6.0 and 40% MPD at 293 K (Fig. 2). Although the average crystals were small, about 15 µm in diameter, the best samples of diameter 30 µm diffracted to a maximum resolution of 1.68 Å in-house (Fig. 3), suggesting a low solvent content. Interestingly, OCD could be co-crystallized in the presence of the substrate L-ornithine or the product L-proline under the established crystallization conditions. Protein crystals formed in the presence of 2–8 m*M* L-ornithine or 2–8 m*M* L-proline. The habit of these crystals did not change significantly compared with the apo form, although crystals grown with ornithine appeared to triple in diameter and appeared more frequently than those grown in the presence of proline.

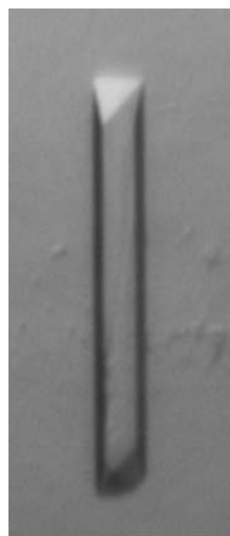

Figure 2
Representative crystal of OCD grown at 293 K from solutions of 0.1 *M* MES pH 6.0 containing 40%(*v*/*v*) MPD. The dimensions of the crystal are approximately 110 × 15 × 15 µm.

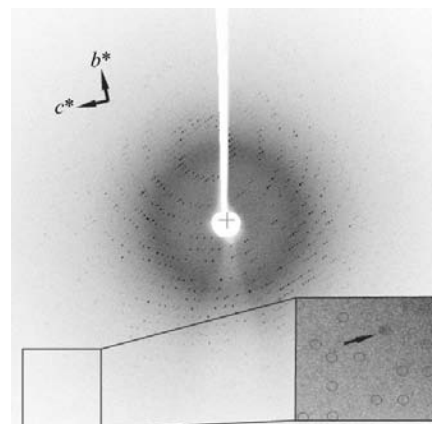

Figure 3
Representative 0.5° oscillation collected from an OCD crystal. The corner of the image plate corresponds to 1.68 Å resolution. The inset box is enlarged and the arrow indicates diffraction at 1.84 Å resolution. The diffraction data were recorded for 20 min at a distance of 150 mm.

3.3. X-ray diffraction data

Crystals of apo OCD diffracted X-rays to 1.68 Å resolution on our in-house source. These data were integrated and scaled, revealing an $I/\sigma(I)$ value of 2.1 in the 1.72–1.68 Å resolution shell. However, complete X-ray diffraction data were recorded to 1.80 Å resolution, which represents the edge of the image plate at 150 mm (Fig. 3). Hence, preliminary results indicated high-resolution data can be obtained, which should be relevant to future studies including refinement of substrate and product complexes.

The space-group assignment of OCD crystals was facilitated by the software package *CrystalClear*, which suggested a primitive orthorhombic Bravais lattice with unit-cell parameters a = 70.0, b = 78.3, c = 119.4 Å. An analysis of the Laue symmetry was consistent with $2/m$ reciprocal-lattice symmetry along each of the principal axes of the major zones $hk0$, $h0l$ and $0kl$ (Fig. 3). Recorded systematic absences of the form $2n + 1$ from the $h00$, $0k0$ and $00l$ reflection classes indicated the presence of 2_1 screw axes indicative of space group $P2_12_12_1$. The resulting Matthews coefficient V_M (Matthews, 1968) was 2.1 Å^3 Da^{-1} (solvent content 42%), corresponding to two 38.5 kDa monomers per asymmetric unit.

Alanine dehydrogenase (AlaDH) from *A. fulgidus* was recently identified as a novel member of the μ-crystallin protein family (Smith *et al.*, 2003) and exhibited 30% sequence identity to the 350-amino-acid polypeptide chain of OCD. Nonetheless, we were unable to obtain a molecular-replacement solution using this molecule as a search model. Inspection of native Patterson maps and self-rotation functions revealed no obvious packing peaks or non-crystallographic symmetry axes, although the latter could be masked by the crystallographic symmetry axes. At present, there are no other known proteins with structural similarity to ornithine cyclodeaminase in the Protein Data Bank. Ultimately, the structure of OCD should provide insight into the fold of this novel protein family. The ability to prepare crystals in the presence of substrates and products under low ionic strength conditions should prove useful in mechanistic studies. An experimental phasing approach has been undertaken in order to solve the OCD structure.

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