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Purification, crystallization and preliminary X-ray study of orotidine 5′-monophosphate decarboxylase

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Orotidine-5′-monophosphate decarboxylase (ODCase) from *Methanobacterium thermoautotrophicum* has been crystallized with and without the inhibitor 6-azaUMP by the vapour-diffusion method. In the absence of the inhibitor, the protein crystallizes in space group $P4_12_12$ (unit-cell parameters $a = b = 56.9$, $c = 124.5$ Å) with one molecule per asymmetric unit; the crystals diffract to 1.8 Å resolution. In the presence of the inhibitor, the protein crystals are monoclinic, space group $P2_1$ (unit-cell parameters $a = 73.0$, $b = 98.6$, $c = 73.3$ Å, $\gamma = 104.0°$), with four molecules in the asymmetric unit; the crystals diffract to 1.5 Å resolution.

1. Introduction

Orotidine 5′-monophosphate decarboxylase (ODCase) catalyzes the conversion of orotidine 5′-monophosphate (OMP) to uridine 5′-monophosphate (UMP), the last step in the *de novo* biosynthesis of pyrimidine nucleotides (Lieberman *et al.*, 1955). ODCase exists as an independent protein in microorganisms and as a part of the bifunctional enzyme UMP synthase in mammals. Unlike other decarboxylation reactions, the enzyme enhances the reaction rate by 10^{17}-fold in the absence of any known cofactors or metal ions (Cui *et al.*, 1999; Miller *et al.*, 1999; Smiley & Saleh, 1999), leading to a second-order rate constant $[k_{cat}/(K_m \times k_{non})]$ of 2.0×10^{23} M^{-1} for the yeast enzyme (Radzicka & Wolfenden, 1995). How this enzyme achieves such remarkable rate enhancement has been under continuous investigation. Two strong inhibitors are known for this enzyme: 6-azauridine 5′-phosphate (6-azaUMP), with an inhibition constant (K_i) of 5.1×10^{-7} M, and 1-(5′-phospho-β-D-ribofuranosyl)barbituric acid (BMP), with a K_i of 8.8×10^{-12} M (Levine *et al.*, 1980). Both are hypothesized to be transition-state analogues. As part of a Structural Genomics Initiative (Edwards & Arrowsmith, 1999), we have crystallized the ODCase from *M. thermoautotrophicum* both in its free form and with 6-azaUMP bound.

2. Materials and methods

2.1. Cloning, expression and purification of ODCase

The full-length ODCase gene (Genebank accession number AE000802) from the genomic DNA of the thermophilic archaeon *M. thermoautotrophicum* ΔH was amplified and cloned into the pET15b vector (Novagen) at the *Nde*I and *Bam*HI sites. The 5′ primer (GCGGCGGCCCATATGTTGAGATCCCG-GAGAGT) and the 3′ primer (GCGCGG-ATTCTCAGGGATTCAGAAGGTCTTT) were designed with *Nde*I and *Bam*HI restriction sites, respectively. The PCR product was gel purified and enzymatically digested with *Nde*I and *Bam*HI (NEB) before ligating into the pET15b vector. The ODCase with an N-terminal His$_6$ tag was then expressed in BL21(DE3)/magic *Escherichia coli* cell strain. The magic plasmid codes for three rare-triplet tRNAs (AGG for Arg, AGA for Arg and ATA for Ile) were controlled by a T7 promoter and a kanamycin-resistance gene. The cells were grown at 310 K in 2 l Luria–Bertani broth supplemented with carbenicillin and kanamycin to an OD$_{600}$ of 0.7 and were then induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 300 K overnight. The cells were spun down at 4000 rev min^{-1} for 30 min and the pellet was resuspended in buffer (5 mM imidazole, 50 mM HEPES pH 7.5, 500 mM NaCl, 5% glycerol) and lysed using a French press. After centrifuging at 17 000 rev min^{-1} for 1 h, the supernatant was loaded onto a 3 ml Ni–NTA column (Qiagen) by gravity. The column was then washed with 100 ml of wash buffer (30 mM imidazole, 50 mM HEPES pH 7.5, 500 mM NaCl, 5% glycerol) and the protein was eluted in 2 ml fractions with elution buffer (250 mM imidazole, 50 mM HEPES pH 7.5, 500 mM NaCl, 5% glycerol). Fractions containing concentrated protein were collected and diluted with buffer (50 mM Tris pH 7.5, 150 mM NaCl, 2.5 mM CaCl$_2$) to 0.5 mg ml^{-1} for the N-terminal His$_6$-tag elimination. 0.5 units of thrombin (Calbiochem) were used for every 0.5 mg of protein. The digest was stopped by
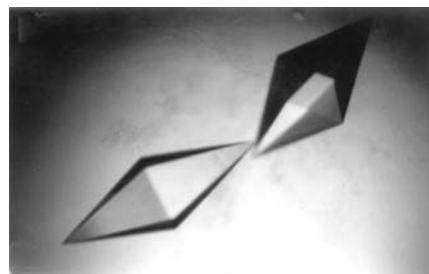
Table 1
Data-collection statistics.

Values in parentheses refer to the outer shell. The outer shell is 1.86–1.80 Å for native apoE, 1.58–1.50 Å for E-azaUMP and 2.07–2.00 Å for Se-Met apoE.

	Native		MAD (Se-Met) of apoE		
	ApoE	E-azaUMP	Remote	Peak	Edge
Wavelength (Å)	1.0	1.0	0.9575	0.9799	0.9802
Resolution (Å)	1.8	1.5	2.0	2.0	2.0
Temperature (K)	100	100	100	100	100
Mosaicity	0.37	0.36	0.5	0.5	0.5
Measured reflections	330779	1127371	417754	399788	400747
Unique reflections	19557	156498	14614	14514	14526
Completeness (%)	98.9 (99.7)	95.6 (87.9)	99.4 (95.3)	98.6 (90.0)	98.6 (89.0)
R_{sym}† (%)	3.8 (7.6)	5.8 (32.6)	6.0 (16.5)	7.0 (13.4)	8.0 (15.7)
Average $I > \sigma(I)$	45.8 (36.9)	8.9 (2.3)	34.7 (9.5)	38.3 (12.5)	36.9 (11.1)
Space group	$P4_12_12$	$P2_1$	$P4_12_12$	$P4_12_12$	$P4_12_12$

† $R_{sym} = \sum |I - \langle I \rangle| / \sum I$, where I is the observed intensity and $\langle I \rangle$ is the average intensity from multiple observations of symmetry-related reflections.

adding PMSF to a final concentration of 1 mM. The mixture was then passed through a second Ni–NTA column by gravity to eliminate undigested protein and the His$_6$ tag. The flow-through was concentrated and buffer-exchanged on a Superdex 200 column (Pharmacia), which was pre-equilibrated with 20 mM HEPES pH 7.5, 5 mM DTT, 150 mM NaCl. The apparent molecular weight derived from the elution time of the protein standards from the size-exclusion column was about 43 kDa.

For the expression of selenomethionine (Se-Met) protein, the vector was trans-formed into a methionine auxotroph strain B834(DE3) (Novagen) along with the magic plasmid. The protein was expressed using M9 media enriched with selenomethionine and the other 19 amino acids (Rama-krishnan *et al.*, 1993). It was purified under the same conditions as the native protein.

2.2. Preliminary activity assay

ODCase enzymatic activity was monitored using a continuous spectro-photometric assay at 285 nm, following the disappearance of OMP (Lieberman *et al.*, 1955). The temperature optimum was determined as 328 K, which is also the growth temperature of *M. thermo-autotrophicum*; therefore, all kinetic assays were carried out at this temperature. To further characterize ODCase, the effects of different metal ions (Mg, Ca and Zn) and of pH were studied. None of the metal ions had any effect on ODCase activity and its optimum activity was observed at pH 7.0.

2.3. Crystallization

An initial crystal growth condition was obtained from Crystal Screen II (Hampton Research), using the hanging-drop vapour-diffusion method. It was then refined by varying the salt concentration and the pH. 2 µl of an 11 mg ml^{-1} protein solution was mixed with an equal volume of well solution containing 1.1 M trisodium citrate pH 6.5, 3% glycerol and was equilibrated for 2–3 weeks. The Se-Met crystal setups were the same except that trisodium citrate pH 7.0 was used. Both types of crystals grew to an ideal size of 0.7 × 0.4 × 0.4 mm within three weeks (Fig. 1a).

6-Azauridine 5′-monophosphate was synthesized from 6-azauridine (Sigma) according to the method of Brody &

Figure 1
(a) Crystals of the free *M. thermoautotrophicum* ODCase were grown from hanging drops with 1.1 M trisodium citrate as precipitant at room temperature. The longest dimension is approximately 0.7 mm. (b) Crytals of the ODCase–6-azaUMP complex were grown under similar conditions but from sitting drops. The longest single needle is about 0.1 mm thick.

Westheimer (1979) with slight modifications. ODCase–6-azaUMP complex crystals were grown from vapour-diffusion sitting drops with 1.2 M trisodium citrate, 0.1 M MES pH 6.5, 5% glycerol as the reservoir. 5 µl of a 10 mg ml^{-1} protein solution containing 10 mM 6-azaUMP was mixed with an equal volume of the reservoir solution and then equilibrated against 0.5 ml of reservoir for two weeks (Fig. 1b).

3. Diffraction measurements

All data collections were performed at 100 K; cryoprotectants were the respective reservoir solutions containing an additional 15% glycerol. Three 2 Å MAD data sets were collected on the Se absorption edge (remote at 0.9575 Å, peak at 0.9799 Å and edge at 0.9802 Å) at beamline 14D BioCARS, APS. A 1.8 Å native data set was collected at beamline 14C BioCARS, APS. The data were processed with *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). A 1.5 Å data set of the ODCase–6-azaUMP complex crystal was collected at beamline 14C BioCARS, APS and was processed with *MOSFLM* (Leslie, 1996).

4. Results and discussion

Typically, a 2 l culture yields 30 mg of pure protein. Preliminary kinetic studies of the enzyme from the thermophilic organism *M. thermoautotrophicum* gave a k_{cat} of 43 s^{-1} at 238 K and of 5.4 s^{-1} at 298 K as well as a K_m of less than 10 µM, compared with a k_{cat} of 39 s^{-1} and a K_m of 0.7 µM for the yeast enzyme at 298 K (Radzicka & Wolfenden, 1995), establishing a similar degree of proficiency for the microbial enzyme.

The Se-Met protein was purified within 24 h of harvesting the cells. 6 M HCl hydrolysis amino-acid analysis indicated 95% incorporation of Se-Met. Soaking of the apoE crystals with the cryosolution containing 10 mM 6-azaUMP resulted in crystal cracking. In addition, the fact that the complex crystallizes under almost the same conditions as the apoE but in a different space group indicates that the binding of the inhibitor induces changes in the enzyme conformation. The complex crystals can also be grown from hanging drops; however, they show a strong tendency to twin in this setup. The degree of twinning and the thickness of these crystals are very sensitive to the trisodium citrate concentration. To obtain usable crystals, larger sitting drops were used. It was then possible to cut off single chunks of the twinned crystals for diffraction analysis.
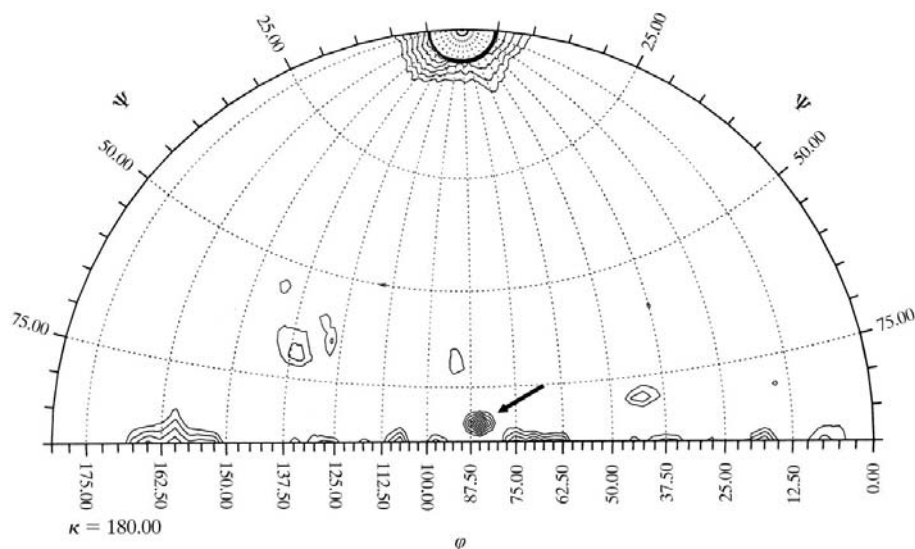
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Figure 2
$\kappa = 180°$ section of the self-rotation function calculated with the complex data set using *GLRF*. The resolution of the data used was 10–3 Å, with a radius of integration of 18 Å. The marked peak ($\psi = 85, \varphi = 85°$) with a height of 4.2σ (25% of the origin peak) indicates a non-crystallographic twofold rotation axis.

The space group of the crystal of the free enzyme was $P4_{1}2_{1}2$ (unit-cell parameters, a, b = 56.9, c = 124.5 Å), with one molecule in the asymmetric unit according to the Matthews coefficient (V_m = 2.0 Å^3 Da^{-1}; Matthews, 1968). The complex crystals belong to space group $P2_1$, with unit-cell parameters a = 73.0, b = 98.6, c = 73.3 Å, α = 90, β = 104.0, γ = 90°, and there are four molecules in the asymmetric unit (V_m = 2.6 Å^3 Da^{-1}). The data sets are more than 95% complete with a good R_{sym} (Table 1).

A self-rotation function was calculated using *GLRF* (Tong & Rossmann, 1997) from the data set obtained from the complex crystals in order to identify possible fourfold or twofold non-crystallographic symmetry. There is only one significant twofold rotation axis close to the c^* axis (Fig. 2), while the self-rotation function only shows noise in the $\kappa = 90°$ plane. This is consistent with the biological observations that yeast ODCase forms functional dimers. A full structural analysis based on

the MAD phasing technique is now under way.

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