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Crystallization and preliminary crystallographic analysis of 3-carboxy-*cis,cis*-muconate lactonizing enzyme from *Neurospora crassa*

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Abstract

Crystals of 3-carboxy-*cis,cis*-muconate lactonizing enzyme (CMLE; E.C. 5.5.1.5) from *Neurospora crassa* that diffract to high resolution have been obtained. The crystals belong to the orthorhombic space group $P2_12_12_1$ with unit-cell dimensions $a = 92.1$, $b = 159.7$, $c = 236.6$ Å (at 103 K) and diffract at most to 2 Å resolution. The asymmetric unit of the crystals appears to contain two tetrameric CMLE molecules making up a total of 328 kDa per asymmetric unit. Both cross-linking with glutaraldehyde and cryo-cooling to 103 K have been used to facilitate data collection because the crystals are unstable in the X-ray beam; both techniques extend the crystal lifetime but cryo-cooling, unlike glutaraldehyde cross-linking, does not lower the quality of the diffraction pattern.

1. Abbreviations

CMLE, 3-carboxy-*cis,cis*-muconate lactonizing enzyme; MLE, *cis,cis*-muconate lactonizing enzyme; CCM, *cis,cis*-muconate; 3-carboxy-CCM, 3-carboxy-*cis,cis*-muconate; TCA, tricarboxylic acid; Cl-MLE, chloromuconate cycloisomerase; β-ME, β-mercaptoethanol; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PEG, polyethylene glycol.

2. Introduction

3-carboxy-*cis,cis*-muconate lactonizing enzyme (CMLE) (also called 3-carboxy-*cis,cis*-muconolactone cycloisomerase) catalyzes the reversible γ-lactonization of 3-carboxy-*cis,cis*-muconate (3-carboxy-CCM). This reaction produces a lactone intermediate in the β-ketoadipate pathway (Stanier & Ornston, 1973), a catabolic route by which bacteria, yeast, and fungi metabolize aromatic compounds to TCA cycle components. In both eukaryotes and prokaryotes, the cyclization reactions of *cis,cis*-muconate (CCM) and 3-carboxy-CCM are catalyzed by muconate lactonizing enzyme (MLE) and CMLE in separate but parallel branches of the pathway.

Despite the apparent similarity of the cycloisomerase reactions, recent studies have suggested the existence of several different classes of these enzymes. Stereochemical analysis of the cyclization reactions and the molecular cloning of bacterial MLE and CMLE have demonstrated that these enzymes lack a common evolutionary origin and, in fact, utilize totally distinct mechanistic motifs. Bacterial MLE catalyzes a metal-dependent *syn* addition, while bacterial CMLE catalyzes an *anti* addition and is related to the class II fumarase family of enzymes based on sequence homology (Williams *et al.*, 1992).

Eukaryotic CMLE's are notably distinct from their prokaryotic counterpart. The stereochemical and regiochemical course of the lactonization catalyzed by CMLE from *N. crassa* is opposite to that of bacterial CMLE and identical to that of bacterial MLE. Nonetheless, *N. crassa* CMLE appears to represent a unique solution to the problem of catalyzing *syn* addition; it does not have sequence homology to *Pseudomonas putida* MLE and does not require a divalent metal for activity (Mazur, Henzel, Mattoo & Kozarich, 1994). However, *Neurosporra crassa* CMLE does show sequence homology to MLE from *Trichosporon cutaneum*, suggesting that the eukaryotic cycloisomerase represent a third cycloisomerases class which has arisen convergently with the bacterial MLE's (Mazur, Pieken *et al.*, 1994), and could represent a yet undiscovered structural motif among the cycloisomerase family.

We have previously determined the crystal structure of *P. putida* MLE (Goldman, Ollis & Steitz, 1987) and subsequently refined it to 1.85 Å (Helin, Kahn, Guha, Mallows & Goldman, 1996). It is an α/β-barrel enzyme which shares extensive structural and mechanistic similarity with mandelate racemase (Neidhart *et al.*, 1991), which is thought to be evolutionarily related to MLE. The structure of *Alcaligenes eutrophus* chloromuconate cycloisomerase (Cl-MLE) (an α/β-barrel enzyme) has been solved by molecular replacement using MLE as the search model (Hoier *et al.*, 1994). Structures for several other enzymes of the β-ketoadipate pathway exist: *P. aeruginosa* protocatechuate 3,4-dioxygenase (a mixed β-barrel enzyme) (Ohlendorf, Lipscomb & Weber, 1988), *P. putida* muconolactone isomerase (a unique decameric enzyme) (Katti, Katz & Wyckoff, 1989), and *Pseudomonas sp.* B13 dienelactone hydrolase (an α/β hydrolase fold structure) (Pathak & Ollis, 1990; Ollis *et al.*, 1992).

The evolutionary relationships among both cycloisomerases and the enzymes of the β-ketoadipate pathway are complex and still largely unresolved. Following the crystallization of *N. crassa* CMLE we intend to solve its structure by X-ray diffraction, which should clarify the relationship between *N. crassa* CMLE and *T. cutaneum* MLE, and the structural and mechanistic consequences of the convergent evolution of the eukaryotic cycloisomerases and the bacterial MLE's. In the current study we report the first crystallization of a CMLE of eukaryotic origin.

3. Experimental

Recombinant *N. crassa* CMLE was expressed in *Escherichia coli* and purified as described earlier (Mazur, Henzel *et al.*,

Table 1. *Data on crystal forms of CMLE*

Crystal type	Habit with size in mm*	Crystallization conditions†	Space group	Cell dimensions‡ (Å)	Z	Diffraction limit (Å)	V_M** (Å^3 Da^{-1}) [with solvent content (%)]
Type 1	Needles 2 × 0.15 × 0.05	10%(w/v) PEG 6000 50 m*M* Tris–HCl, pH 8.0–8.5 or 20%(w/v) PEG 4000 100 m*M* Tris–HCl, pH 7.5 14%(v/v) 2-propanol	nd§	nd	nd	nd	nd
Type 2	Prisms 0.8 × 0.7 × 0.3	7.5%(w/v) PEG 6000 800 m*M* Li-surface or 20%(w/v) PEG 6000 100 m*M* Na-citrate, pH 5.6 400 m*M* $(NH_4)_2SO_4$	$P2_12_12_1$	$a = 92.9$ $b = 162.3$ $c = 246.6$	8	2.7	2.83 (57)
Type 2*b*	Prisms 1.5 × 1.0 × 0.4	1.56 *M* $(NH_4)_2SO_4$ 100 m*M* PIPES,¶ pH 5.7 at 277 K	$P2_12_12_1$	$a = 92.9$ $b = 162.3$ $c = 246.6$	8	2.0	2.83 (57)

* The best size achieved is reported. Usual crystal sizes equal to about one-half in each cell edge for all crystal forms. † The temperature used is 294 K if not otherwise stated. Protein concentration was not found to be critical and was usually between 10 and 20 mg ml^{-1}. ‡ At 294 K. § nd = not determined. ¶ PIPES = piperazine-*N*,*N*′-bis(2-ethanesulfonic acid). ** V_M = crystal density as calculated according to Matthews (1968).

1994). CMLE is a homotetramer in solution with a subunit molecular weight of 41 kDa. The samples were precipitated with ammonium sulfate (75% saturation) in the presence of 50 m*M* Tris–HCl, pH 7.5, 1 m*M* β-ME and 100 m*M* NaCl and stored in the cold. For crystallization experiments the samples were dialyzed against 10 m*M* Tris–HCl, pH 7.5, 1 m*M* β-ME. There were no detectable impurities on overloaded SDS–PAGE gels (Mazur, Henzel *et al.*, 1994).

We used the hanging-drop vapor-equilibration method (McPherson, 1982) to obtain crystals of CMLE. 1 ml precipitant solutions were prepared in the wells of standard 24-well Linbro cell-culture plates. Drops containing the protein were made by mixing equal volumes (3–5 µl) of the protein's stock solution and the precipitant solution from the well.

We used the crystallization strategy of Jancarik & Kim (1991). This empirical method is based on previously successful crystallization conditions found frequently in crystallization literature. We obtained two crystal forms from several different conditions. However, this method does not thoroughly explore the potential of ammonium sulfate as the crystallization agent. Therefore, we set up an independent screening series employing ammonium sulfate as the precipitant, and the best crystals were found in this series. Subsequently, we used a computer-controlled motorized pipette and the PIPEX system (Eiselé, 1993) to fine-tune the crystallization conditions for CMLE.

Data were collected using the R-AXIS IIC image-plate system mounted on a Rigaku RU200 rotating-anode X-ray generator run at 50 kV and 180 mA (Cu $K\alpha$, graphite monochromator, 0.3 mm collimator) and at beamline X4A (wavelength 0.96875 Å) at the National Synchrotron Light Source at Brookhaven National Laboratory, NY, USA.

The unit-cell and space-group data of the CMLE crystals were readily obtained from still photographs processed by the autoindexing procedure of the R-AXIS IIC software (Molecular Structure Corporation, The Woodlands, Texas) or by indexing 1° oscillation frames with the program *DENZO* (Otwinowski & Minor, *The HKL Program Suite*, 1996), and were confirmed by precession photography using an Enraf–Nonius precession camera. The density of the crystals was measured in Ficoll gradients (Mikol & Giegé, 1992).

4. Results and discussion

The conditions leading to optimal crystal size are listed in Table 1. The crystals used for data collection belong to the orthorhombic space group $P2_12_12_1$, have cell dimensions $a = 92.1$, $b = 159.7$, $c = 236.6$ Å (measured at 103 K; Table 2), and grow in 1–2 weeks at 294 K and in 3–6 weeks at 277 K. Crystals grown in the cold are usually bigger and morphologically better. The type 2*b* crystals (Table 1) diffract the best, possibly because they grow slowest. This crystal form was used in all subsequent work. The type 1 crystals are too fragile to be mounted.

CMLE crystals are rather unstable in the X-ray beam. The high-resolution diffraction disappears in less than an hour and data collection from one crystal is usable to 4 Å resolution at best. Therefore, collecting a high-resolution data set would mean using many crystals or a better method of data collection.

We have collected data on crystals cryo-cooled to 103 K (Hope, 1988; Teng, 1990; Gamblin & Rodgers, 1993). The best cryo-protectant is polyethylene glycol 400 (PEG 400). We replaced the ammonium sulfate in the mother liquor by PEG 400 [40%(w/v) final concentration] and incubated the crystals in this solution in the cold for seven to ten minutes prior to flash-freezing. Crystals, when frozen, diffract to high resolution indefinitely. The volume of the unit cell shrinks by 6.4% upon cryo-cooling (Table 2). Cross-linking the crystals (Quiocho & Richards, 1964; Kasvinsky & Madsen, 1976) in 3% glutaraldehyde for 3 h prior to mounting also improves the crystal lifetime considerably but does not permit high-resolution data collection. The data would nevertheless be usable.

Given that CMLE is a homotetramer in solution, we calculated the V_M value for the crystals (Matthews, 1968) assuming one or two tetramers per asymmetric unit (a.u.). Assuming one tetramer per a.u. gives a value of 5.67 and assuming two, a value of 2.83. They correspond to solvent

Table 2. *Effect of cryo-cooling and glutaraldehyde cross-linking on unit-cell dimensions of the CMLE crystals*

	Crystals without stabilizing treatment	Cryo-cooled crystals	Cross-linked crystals
a (Å)	92.9	92.1	93.1
b (Å)	162.3	159.7	161.1
c (Å)	246.6	236.6	249.1

contents of 78 and 57%, respectively. This suggests that it is likely that the unit cell contains two tetramers. We measured the crystal density by flotation in Ficoll gradients (Mikol & Giegé, 1992) and obtained a solvent content of 57.1 (1.7)% at 294 K, consistent with two tetramers per asymmetric unit. Rotation function searches for non-crystallographic two- and fourfolds were, however, inconclusive – a perhaps not unsurprising result given the presence of up to ten different twofold axes: three crystallographic screw axes that appear as twofolds in Patterson space and up to seven non-crystallographic twofolds (three relating the monomers in each possible D_2 tetramer and one inter-tetramer twofold).

We have collected data from cryo-cooled crystals to a nominal resolution of 2 Å at the National Synchrotron Light Source at Brookhaven National Laboratory. Data sets on native crystals, several potential heavy-atom derivatives and crystals soaked in substrate or inhibitor solutions were collected. Evaluation of the data is in progress and will be published elsewhere.

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