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Cocrystallization and preliminary crystallographic analysis of an inactive MaoC-like hydratase mutant with the substrate crotonyl-CoA

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MaoC-like hydratase (MaoC) is a recently identified enzyme involved in the biosynthetic pathway of polyhydroxyalkanoates (PHAs), which are completely biodegradable polymers used to produce green plastics. The inactive mutant D194N-MaoC was crystallized in the presence of the substrate crotonyl-CoA. Crystals were grown in a number of conditions, but only those produced using 20%(*v*/*v*) ethylene glycol were suitable for structural studies. Data were collected to 2.10 Å resolution using X-radiation and the crystal belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 81.40$, $b = 82.58$, $c = 123.99$ Å.

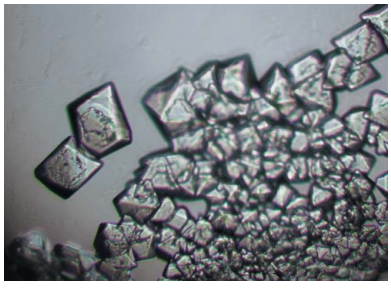
1. Introduction

MaoC-like hydratase (MaoC), which has (*R*)-specific enoyl-CoA hydratase [(*R*)-hydratase] activity and is involved in linking the β-oxidation and polyhydroxyalkanoate (PHA) biosynthetic pathways, has recently been identified in the *fadB* mutant *Escherichia coli* strain (Park & Lee, 2003). (*R*)-Hydratases catalyze an *R*-specific hydration reaction of the fatty-acid β-oxidation intermediates 2-*trans*-enoyl-CoAs to (*R*)-3-hydroxyacyl-CoAs and play an important role in supplying monomer units from β-oxidation to PHA synthesis (Tsuge, Taguchi *et al.*, 2003).

PHAs are 100% biodegradable polymers with biocompatible, thermoplastic and mechanical properties and have attracted much attention in recent years as ideal substitutes for petrochemical-derived plastics, packaging and biomedical materials (Madison & Huisman, 1999). With global environmental problems becoming more and more serious, it is essential to develop environmentally protective or green alternatives to conventional materials and therefore much research has focused on establishing the efficient production of PHAs (Tsuge, Hisano *et al.*, 2003). However, commercial PHA production is limited by high cost. Cutting the cost of PHA production will make PHA competitive as a green alternative to conventional plastics, which are difficult to degrade (Lee & Choi, 1998).

Many enzymes have been identified in the PHA-biosynthesis pathway, including (*R*)-hydratases, acyl-CoAs, enoyl-CoAs, (*S*)-3-hydroxyacyl-CoAs *etc.* (Lu *et al.*, 2008). (*R*)-Hydratases are essentially involved in the biosynthesis of PHA, functioning as a monomer-supplying enzyme (Fukui & Doi, 1997). To date, many organisms have been found to possess (*R*)-hydratase either as a monofunctional enzyme or as an (*R*)-hydratase domain (hydratase 2 domain) of a multifunctional enzyme (Tsuge, Hisano *et al.*, 2003; Tsuge, Taguchi *et al.*, 2003). MaoC is a newly identified member of the (*R*)-hydratases which has been employed to supply the PHA precursors (*R*)-3-hydroxyacyl-CoAs (Park & Lee, 2003). Several structures of (*R*)-hydratases have been resolved and we have determined the structure of MaoC from *Phytophthora capsici*. However, attempts to prepare crystals of MaoC with substrates have been unsuccessful until now.

In this study, we obtained the inactive MaoC mutant D194N-MaoC by site-directed mutagenesis and crystallized the purified mutant in the presence of the substrate crotonyl-CoA. Crystals were finally grown using 20%(*v*/*v*) ethylene glycol, which differed from the



condition that produced native MaoC crystals (Wang *et al.*, 2010). This is the first report of MaoC crystallized in the presence of the substrate crotonyl-CoA and determination of the potential complex crystal structure will provide a better understanding of its probable catalytic activities, in particular the substrate-binding model.

2. Materials and methods

2.1. Construction of the expression plasmid

The MaoC-like hydratase gene (*maoC*; GenBank No. GU190361) from *P. capsici* was cloned as described previously (Wang *et al.*, 2010). Site-directed mutagenesis was performed by the PCR method and was confirmed by DNA sequencing. The primers used for the amino-acid substitution of Asp194 to Asn were sense, 5′-GTCGGGC**AAC**-TACAACTC-3′, and antisense, 5′-GAGTTGTA**GTT**GCCCGAC-3′ (the sequences in bold indicate the mutation site). The mutant product was cloned into pET-28a (Novagen), a vector that adds an N-terminal His_6 tag to the expressed protein, *via* *Eco*RI/*Bam*HI restriction sites. The gene encoding D194N-MaoC with 34 additional residues (MGSSHHHHHHSSGLVPRGSHMASMTGGQQMGR-GS) was transformed into *E. coli* strain BL21 (DE3). The tag was not removed during the purification and crystallization steps.

2.2. Cocrystallization

The expression and purification of the D194N-MaoC mutant was performed as described previously for the wild-type protein (Wang *et al.*, 2010). Protein concentrations were confirmed spectrophotometrically by the Bradford method (Bradford, 1976). The purified D194N-MaoC was concentrated to 20 mg ml^{-1} (about 0.5 m*M*) by ultrafiltration using Amicon Ultra centrifugal filters (10 kDa cutoff; Millipore) and stored in 10 m*M* Tris–HCl pH 8.3 buffer. The substrate crotonyl-CoA was dissolved to 10 m*M* concentration in 10 m*M* Tris–HCl pH 8.3 buffer (the same buffer as for the purified D194N-MaoC stock solution). Prior to cocrystallization, 80 µl purified D194N-MaoC was mixed with 20 µl dissolved crotonyl-CoA, corresponding to a 1:5 molar ratio of the protein and the substrate crotonyl-CoA. Initial crystallization trials were performed manually with Crystal Screen, Crystal Screen 2 and PEG/Ion (Hampton Research) using the hanging-drop vapour-diffusion technique at 283 K in 16-well Linbro plates. The drops consisted of 1 µl protein–crotonyl-CoA solution and an equal volume of screening solution and were equilibrated against 400 µl reservoir solution. Crystals were obtained after 2 d in (i) 100 m*M* HEPES pH 7.5, 10%(*w*/*v*) polyethylene glycol (PEG) 6000, 5%(*v*/*v*) (±)-2-methyl-2,4-pentanediol and (ii) 25%(*v*/*v*) ethylene glycol. The first condition was the same as that used to obtain native MaoC crystals. Optimization of the crystallization conditions using different PEG, (±)-2-methyl-2,4-pentanediol and ethylene glycol concentrations was performed to obtain crystals that were suitable for data collection. The crystals (Fig. 1) used for the final data collection grew within 2 d using 3 µl drops that consisted of 1.5 µl of the protein–crotonyl-CoA solution specified above and 1.5 µl reservoir solution consisting of 20%(*v*/*v*) ethylene glycol and were equilibrated against 400 µl reservoir solution.

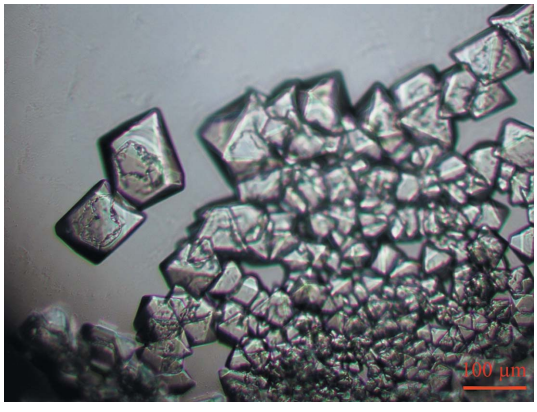


Figure 1
Crystals of the D194N-MaoC mutant in the presence of the substrate crotonyl-CoA.

2.3. Data collection

Initial screening for X-ray diffraction using an in-house X-ray generator (Cu $K\alpha$ X-rays, Bruker MicroStar) at Shandong University showed that only the crystals obtained using 20%(*v*/*v*) ethylene glycol exhibited a good diffraction pattern. For data collection, crystals were flash-cooled to 100 K after soaking in a cryoprotectant solution consisting of reservoir solution supplemented with 20%(*v*/*v*) glycerol. A complete data set was collected using the same X-ray generator and an image-plate detector. The crystal-to-detector distance was 175 mm and the oscillation range was set to 1.0°.

3. Results and discussion

Several crystal structures of (*R*)-hydratases have been reported. The structure of (*R*)-hydratase from *Aeromonas caviae* has a so-called 'hot-dog' fold which contains the hydratase 2 motif residues (Hisano *et al.*, 2003), in which Asp31 and His36 form a catalytic dyad. Another active-site residue Gly533 was described in the crystal structure of human Hs-H2 (Koski *et al.*, 2005) and these amino acids were found to be highly conserved in various (*R*)-hydratases (Hisano *et al.*, 2003). Our previous study indicated that the catalytically important residues of MaoC-like hydratase from *P. capsici* are Asp194, His199 and Gly217 and this was further confirmed by mutagenesis (unpublished

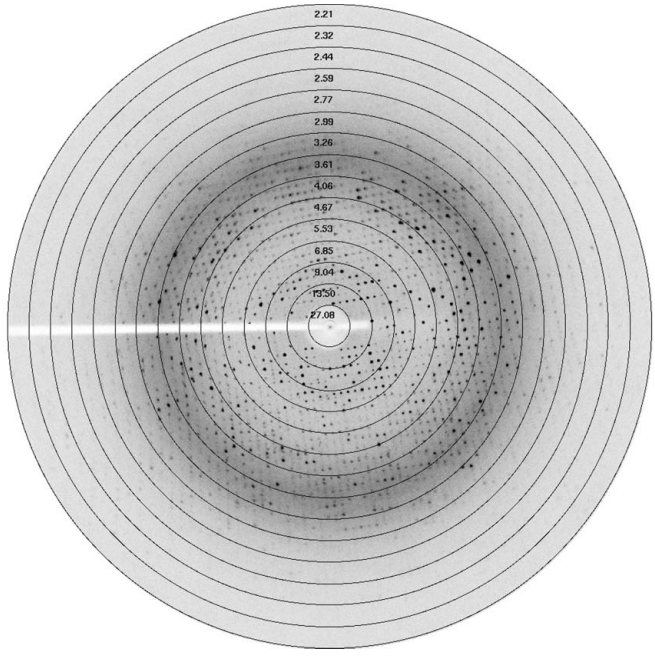


Figure 2
Diffraction pattern of a crystal of the D194N-MaoC mutant in the presence of the substrate crotonyl-CoA. The resolution rings are shown.

Table 1
Crystal data and data-collection statistics.

Values in parentheses are for the highest resolution shell.

Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 81.40, b = 82.58, c = 123.99
No. of protomers per asymmetric unit	2
Temperature (K)	100
Wavelength (Å)	1.54
Oscillation range (°)	1.0
Crystal-to-detector distance (mm)	175
No. of frames	180
Resolution range (Å)	50–2.10 (2.18–2.10)
Completeness (%)	93.1 (86.4)
$R_{\rm merge}$† (%)	11.4 (58.2)
Mean $I/\sigma(I)$	16.98 (2.3)
Multiplicity	5.8 (4.7)
Wilson B factor (Å^2)	38.4

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the weighted average intensity for all observations of reflection hkl.

work). We chose the inactive mutant D194N-MaoC for the cocrystallization study with the substrate crotonyl-CoA.

The D194N-MaoC mutant was successfully overexpressed in *E. coli* strain BL21 (DE3) and the yield of protein was about 50 mg per litre of culture. The expressed products were purified using Ni-Sepharose affinity chromatography and gel-filtration chromatography and the final protein purity was greater than 95% as analysed by SDS–PAGE, showing a significant amount of a 36 kDa protein containing a six-His tag at the N-terminus, which was in agreement with the predicted molecular weight of the mutant. Crystals grown using the same condition as used for native MaoC did not diffract sufficiently and crystals suitable for data collection were only obtained using 20%(*v*/*v*) ethylene glycol. It was not necessary to dehydrate the crystals as was essential to obtain high diffraction-quality native MaoC crystals. However, the glycerol concentration in the cryoprotectant solution was important to obtain good diffraction data and the best glycerol concentration was found to be 20%(*v*/*v*) after repeated comparisons and experiments. Diffraction data without split spots were obtained from the crystal in the resolution range 50–2.10 Å (Fig. 2) and were processed using the *HKL*-2000 program package (Otwinowski & Minor, 1997). Data-collection and processing statistics are shown in Table 1. The crystals belonged to space group $P2_12_12_1$, with unit-cell parameters a = 81.40, b = 82.58, c = 123.99 Å. The Matthews coefficient suggests that the asymmetric unit contains two molecules and that the protein is a homodimer. Structure determination will be pursued by the molecular-replacement method using the structure of native MaoC (PDB entry 3kh8; H. Wang, K. Zhang, J. Guo, Q. Zhou, X. Zheng, F. Sun, H. Pang & X. Zhang, unpublished work) as a model.

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