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Crystallization and preliminary X-ray analysis of the MaoC-like dehydratase from *Phytophthora capsici*

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MaoC-like dehydratase (MaoC) plays an important role in supplying (*R*)-3-hydroxyacyl-CoA from the fatty-acid oxidation pathway to polyhydroxyalkanoate (PHA) biosynthetic pathways. PHAs have been attracting much attention as they can be used in the biosynthesis of synthetic plastics. Crystals of MaoC from *Phytophora capsici* were grown by the hanging-drop vapour-diffusion method at 289 K in a number of screening conditions. An MaoC crystal diffracted to 1.93 Å resolution using X-ray radiation and belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 81.458, b = 82.614, c = 124.228 Å, $\alpha = \beta = \gamma = 90^\circ$.

1. Introduction

MaoC-like dehydratase (MaoC) is a newly identified enoyl-CoA hydratase that is involved in linking the β-oxidation and polyhydroxyalkanoate (PHA) biosynthetic pathways (Park & Lee, 2003). PHAs are polyesters of various hydroxycarboxylic acids that are accumulated in a wide variety of bacterial strains. As such, they can be used as an energy-storage and carbon-storage material in the presence of excess carbon source (Madison & Huisman, 1999).

The pathways for the biosynthesis and degradation of PHAs have been well examined in numerous bacteria (Lee, 1996). These include, for example, medium-chain-length PHA-producing bacteria such as *Escherichia coli* (Park & Lee, 2003) and short-chain-length PHA-producing bacteria such as *Ralstonia eutropha* and *Alcaligenes latus* (Schubert *et al.*, 1988). Many metabolic pathways, including fatty-acid β-oxidation and biosynthesis pathways, provide the most favourable substrate for PHA synthase, (*R*)-3-hydroxyacyl-CoA (R3HA-CoA; Madison & Huisman, 1999; Lee, 1996). In pseudomonads which belong to rRNA homology group I, the intermediates of fatty-acid metabolism, including enoyl-CoA, (*S*)-3-hydroxyacyl-CoA, 3-ketoacyl-CoA and 3-hydroxyacyl-acyl carrier protein (ACP), are major precursors of medium-chain-length PHAs (Witholt & Kessler, 1999). MaoC, which has enoyl-CoA hydratase activity and is involved in linking the β-oxidation and the PHA-biosynthetic pathways in *fadB* mutant *E. coli*, has recently been identified (Park & Lee, 2003). However, there are no reports of MaoC in fungi and oomycetes.

PHA has attracted industrial attention for its potential use as a practical degradable thermoplastic (Tsuge *et al.*, 2003). Its biodegradability makes PHA an extremely desirable substitute for synthetic plastics and therefore much research has been focused on establishing its efficient production. Various methods leading to the accumulation of PHA with desirable properties with low loss and high productivity have been developed (Li *et al.*, 2007). A number of bacteria, including *Alcaligenes*, *Pseudomonas*, recombinant *E. coli* and methylotrophs, have been used for the production of PHAs and high productivities have been achieved (Khanna & Srivastava, 2005). Reducing the production costs of PHAs would make them competitive as a 'green plastic' alternative to conventional plastics, which are hard to degrade (Lee & Choi, 1998).

The crystal structure of the (*R*)-specific enoyl-CoA hydratase from *Aeromonas caviae*, which is involved in PHA biosynthesis and shows homology to MaoC, has recently been solved (Hisano *et al.*, 2003). The core structure is a hot-dog fold, which is built up of a long and

hydrophobic α-helix packed against an antiparallel β-sheet. There is about 35% sequence homology between MaoC and 2-enoyl-CoA hydratase 2 (PDB code 1s9c; Koski *et al.*, 2005), which shows the highest sequence homology with MaoC, but the extent of the similarity of their mechanisms remains unknown (Reddy *et al.*, 2003). Structural studies are essential in order to further improve the understanding of the mechanisms of action of these enzymes and to improve and choose better candidates for PHA production.

In this paper, the cloning, expression, purification, crystallization and preliminary X-ray diffraction analysis of the full-length MaoC-like dehydratase (MaoC) from *Phytophora capsici* are described. The crystals reported here will form the basis for the determination of the structure of MaoC and the identification of the amino acids that are associated with the active site, thereby providing a clear view of its probable catalytic activities.

2. Materials and methods

2.1. Protein expression and purification

The MaoC-like dehydratase gene (GenBank No. GU190361) from *P. capsici* was cloned into pET-28a vector (Novagen) *via* *Eco*RI/*Bam*HI restriction sites. The fragment was amplified by polymerase chain reaction (PCR) from the genomic DNA of *P. capsici* (SD33) using sense 5′-CGC**GGATCC**ATGAGTGTGAACGTGGACAAG-3′ and antisense 5′-CCG**GAATTC**TTACAAACGCGCACTGGCGTC-3′ primers (Invitrogen, People's Republic of China). The construct was verified by sequencing and contained a six-His tag at the N-terminus of the protein. *E. coli* strain BL21 (DE3) (Novagen) was used for the expression of MaoC. Cells were grown in lysogeny broth medium (LB) containing 50 mg ml^{-1} kanomycin at 310 K for 3 h. After the addition of 1 m*M* isopropyl β-D-1-thiogalactopyranoside, the culture was grown at 289 K for a further 20 h to induce expression of His_6-tagged protein. Cells were harvested, resuspended in buffer *A* (20 m*M* Tris–HCl pH 8.3 containing 150 m*M* NaCl, 10 m*M* imidazole and 5 m*M* β-mercaptoethanol) and lysed by sonication. After centrifugation, the supernatant was loaded onto an Ni–NTA Superflow column (GE Healthcare) equilibrated with buffer *A* and linked to an ÄKTA Purifier system (GE Healthcare). The column was washed with buffer *B* (buffer *A* containing 40 m*M* instead of 10 m*M* imidazole) and the proteins were eluted with buffer *C* (buffer *A* containing 200 m*M* instead of 10 m*M* imidazole). The solution was concentrated in a centrifugal concentrator (Millipore) and applied onto a Superdex 75 gel-filtration column equilibrated in 10 m*M* Tris–HCl pH 8.3, 50 m*M* NaCl and 5 m*M* DTT. The protein eluting from this column appeared to be greater than 98% pure by SDS–PAGE (not shown) and was dialyzed into 10 m*M* Tris–HCl pH 8.3 and concentrated to 15 mg ml^{-1} prior to crystallization. Reducing agents were included throughout lysis and purification but were not included in the final protein buffer.

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 = 81.458, b = 82.614, c = 124.228, $\alpha = \beta = \gamma$ = 90.0
No. of protomers per ASU	2
Temperature (K)	100
Wavelength (Å)	1.5
Oscillation range (°)	1.0
Crystal-to-detector distance (mm)	140
No. of frames	90
Resolution range (Å)	50–1.93 (1.96–1.93)
Completeness (%)	97.9 (95.5)
R_{merge}† (%)	11.1 (71.5)
Mean $I/\sigma(I)$	19.6 (2.6)
Redundancy	6.7 (5.9)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $\langle I(hkl)\rangle$ is the mean intensity of symmetry-equivalent reflections.

2.2. Crystallization

Initial crystallization conditions were established by the hanging-drop vapour-diffusion technique using 16-well Linbro plates. Purified MaoC was initially crystallized using the sitting-drop vapour-diffusion method at 289 K using 1 µl protein solution and 1 µl screening solution. The Crystal Screen I and Crystal Screen II (Hampton Research) sets of screening conditions were used for initial screening. Small crystals (Fig. 1) were grown in 100 m*M* HEPES pH 7.5, 10%(*w*/*v*) polyethylene glycol (PEG) 6000, 5%(*v*/*v*) (±)-2-methyl-2,4-pentanediol. Optimization of the crystallization conditions was performed by altering the pH, the PEG concentration and the MaoC protein concentration to yield crystals that were suitable for data collection. The final MaoC crystals (Fig. 2) used for data collection were grown in 2 d using 3 µl drops containing 1.5 µl protein and 1.5 µl reservoir solution [100 m*M* HEPES pH 7.5, 6%(*w*/*v*) PEG 6000, 5%(*v*/*v*) (±)-2-methyl-2,4-pentanediol]. The volume of the reservoir solution was 200 µl.

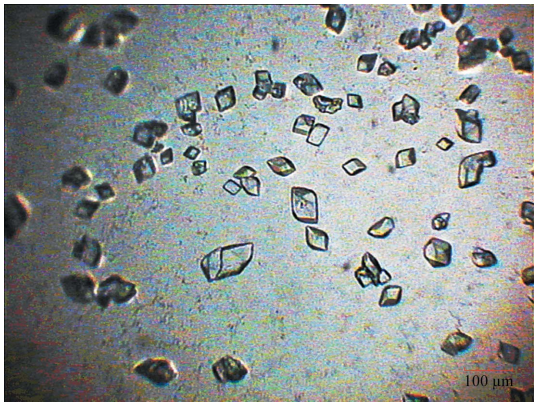

Figure 1
Small crystals obtained by initial screening. The scale bar represents 0.1 mm.

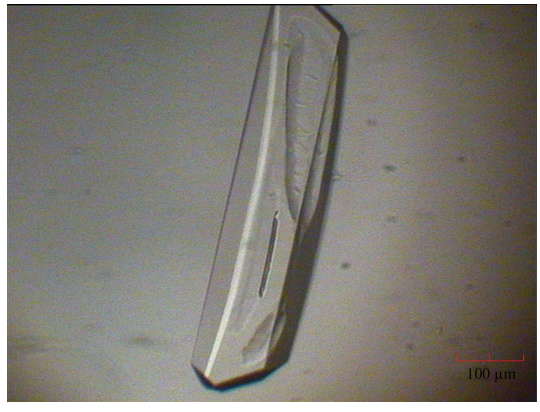

Figure 2
Single crystal of MaoC after optimization of the crystallization conditions. The scale bar represents 0.1 mm.

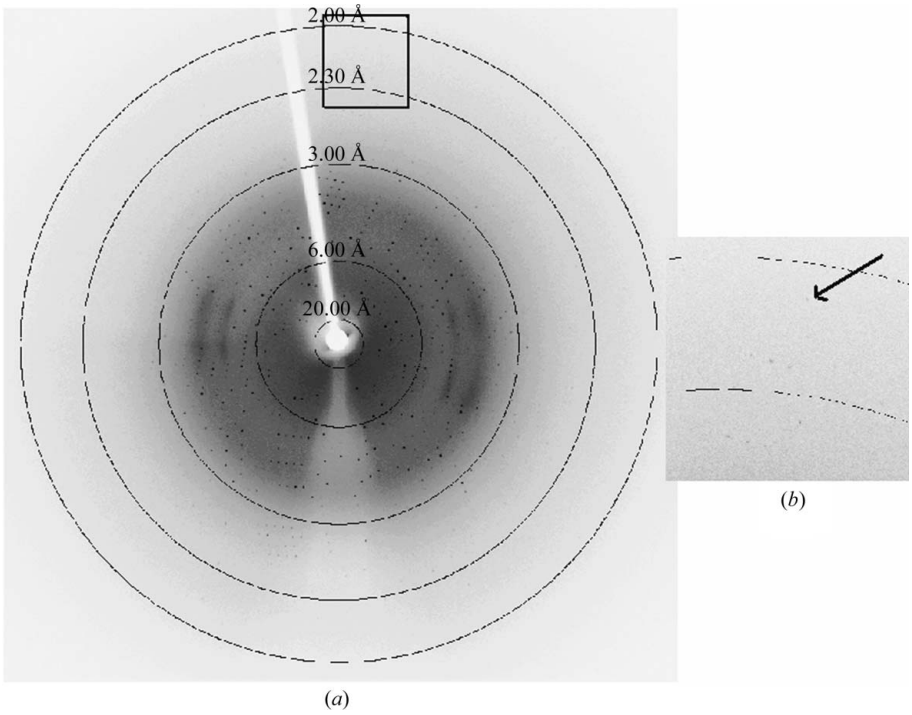

Figure 3
Diffraction pattern of the MaoC crystal. (*a*) A typical diffraction pattern in which the concentric circles indicate resolutions of 20.00, 6.00, 3.00, 2.30 and 2.00 Å. (*b*) An enlarged view of the region marked by a dotted line in (*a*). The arrow indicates a diffraction spot at about 1.93 Å resolution.

2.3. Data collection

Prior to data collection, crystals were dehydrated for 15, 30 or 60 min in a solution containing 12%(*w*/*v*) PEG 6000, 5%(*v*/*v*) (±)-2-methyl-2,4-pentanediol, 20%(*v*/*v*) glycerol, 100 m*M* HEPES pH 7.5, transferred to a cryostream and cooled to 100 K in a nitrogen-gas stream. A complete data set was collected using an in-house (Institute of Biophysics, Chinese Academy of Sciences) rotating-anode FR-E X-ray generator and R-AXIS IV^{++} image-plate detector (Rigaku, Japan). The crystal-to-detector distance was 140 mm and the oscillation range was set to 1.0°. The data were processed using the *CrystalClear* program (Rigaku, Japan).

3. Results and discussion

The probable 897 bp MaoC-like dehydratase gene was successfully PCR-amplified from *P. capsici* genomic DNA. The yield of protein was about 60 mg per litre of culture. Analysis by SDS–PAGE showed 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 this protein. The initial crystals of MaoC (Fig. 1) were too small to obtain good diffraction. Sufficiently large crystals (Fig. 2) were grown after increasing the MaoC protein concentration and reducing the pH of the MaoC protein buffer from 8.5 to 8.3. The use of various cryoprotectant agents such as ethylene glycol, medium-light paraffin oil or sucrose did not improve diffraction. It is important to note that dehydration of the MaoC crystals was a prerequisite for high diffraction quality. The ideal dehydration time was 30 min, while the optimal PEG 6000 concentration for use in dehydration of the crystals was twice that of the mother liquor in which the crystals had grown. Diffraction data were obtained from the crystal in the resolution range 50–1.93 Å (Fig. 3) 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 $a = 81.458$, $b = 82.614$, $c = 124.228$ Å, $\alpha = \beta = \gamma = 90°$. The Matthews coefficient suggested that the asymmetric unit contained two molecules.

Our study describes the expression, purification and preliminary crystallographic analysis of MaoC from *P. capsici*. We intend to solve the structure by molecular replacement using the coordinates of the 2-enoyl-CoA hydratase 2 structure (PDB code 1s9c; Koski *et al.*, 2005) as a search model.

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