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Crystallization and preliminary X-ray diffraction studies of maleylacetate reductase from *Rhizobium* sp. strain MTP-10005

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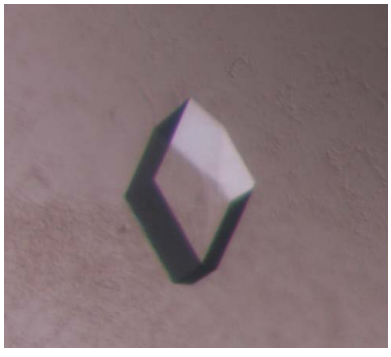

Maleylacetate reductase (EC 1.3.1.32), which catalyzes the reduction of maleylacetate to 3-oxoadipate, plays an important role in the aerobic microbial catabolism of resorcinol. The enzyme has been crystallized at 293 K by the sitting-drop vapour-diffusion method supplemented with a microseeding technique, using ammonium sulfate as the precipitating agent. The crystal belonged to the monoclinic space group *C*2, with unit-cell parameters $a = 56.85$, $b = 121.13$, $c = 94.09$ Å, $\beta = 101.48^\circ$, and contained one dimeric molecule in the asymmetric unit. It diffracted to 1.79 Å resolution.

1. Introduction

Rhizobium is a genus of tubercle-forming bacteria that grow in the root of a plant in symbiosis with other bacteria, such as *Astragalus sinicus*, *Trifolium repens* L. and *Medicago polymorpha* Linn, to fix nitrogen from the air (Beringer *et al.*, 1979). Therefore, much attention has been paid to the *Rhizobium* genes and gene products owing to their role in the regulation of symbiosis. Despite extensive studies on *Rhizobium* (Wardhan *et al.*, 1989; Krishnan & Pueppke, 1991; Harrison *et al.*, 2003), there is still little information available on the molecular structure, function and detailed properties of the enzymes involved in its metabolic pathways.

In the course of a screening experiment, *Rhizobium* sp. strain MTP-10005 was isolated from natural river water as a microorganism with a high level of γ-resorcylate (2,6-hydroxybenzoate) decarboxylase (EC 4.1.1.*x*) activity (Yoshida *et al.*, 2004). During the cloning of the *graF* gene encoding the enzyme γ-resorcylate decarboxylase, the genes *graA, graB* and *graC* were found immediately upstream and downstream of *graF*. They were equivalent to the genes encoding the enzymes in the resorcinol catabolic pathway (Yoshida *et al.*, 2004) that is known in *Pseudomonas putida* (Chapman & Ribbons, 1976) and *Trichosporon cutaneum* (Gaal & Neujahr, 1979) but not in other *Rhizobium* sp. bacteria. Recently, four further genes (*graD*, *graR*, *graE* and *graK*) in the same pathway have been found (Yoshida *et al.*, 2007). Thus, the gene cluster *graRDAFCBEK* has been identified as being involved in γ-resorcylate catabolism by *Rhizobium* sp. strain MTP-10005. Enzymological studies showed that *graD*, *graA*, *graB* and *graC* encode the reductase (GraD) and oxidase (GraA) components of resorcinol hydroxylase (EC 1.14.13.*x*), hydroxyquinol 1,2-dioxygenase (GraB; EC 1.13.11.37) and maleylacetate reductase (GraC; EC 1.3.1.32), respectively. In the aerobic bacterium *Rhizobium* sp. strain MTP-10005, γ-resorcylate is first converted to resorcinol by γ-resorcylate decarboxylase (GraF). Subsequently, resorcinol is sequentially converted into 3-oxoadipate *via* hydroxyquinol and maleylacetate by resorcinol hydroxylase (GraA and GraD), hydroxyquinol 1,2-dioxygenase (GraB) and maleylacetate reductase (GraC).

Recently, the crystal structures of 2,6-dihydroxybenzoate decarboxylase from *Rhizobium* sp. strain MTP-10005 were determined in three forms: in the native form, in complex with the native substrate 2,6-dihydroxybenzoate and in complex with 2,3-dihydroxybenzaldehyde (Goto *et al.*, 2006). Moreover, a catalytic mechanism of 2,6-dihydroxybenzoate decarboxylase involving a novel Zn^{2+}-dependent

decarboxylation has been proposed based on these structures. γ-Resorcylate decarboxylation is the reaction that converts 2,6-dihydroxybenzoate to resorcinol just before the resorcinol catabolic pathway. We have been performing X-ray structural studies of the enzymes, including maleylacetate reductuse (GraC), that are involved in the resorcinol catabolism of *Rhizobium* sp. strain MTP-10005. Despite intensive biological investigations, no crystal structures of maleylacetate reductases from any species have yet been elucidated.

Maleylacetate reductases play a crucial role in the aerobic microbial degradation of aromatic compounds. In fungi and yeasts, the enzymes are involved in the catabolism of very common compounds such as phenol, tyrosine, benzoate, 4-hydroxybenzoate and resorcinol (Karasevich & Ivoilov, 1977; Buswell & Eriksson, 1979; Gaal & Neujahr, 1979; Sparnins *et al.*, 1979; Anderson & Dagley, 1980; Jones *et al.*, 1995). In bacteria, the enzymes contribute to the degradation of resorcinol, 2,4-dihydroxybenzoate (β-resorcylate) and 2,6-dihydroxybenzoate (γ-resorcylate) *via* hydroxyquinol and maleylacetate (Larway & Evans, 1965; Chapman & Ribbons, 1976; Stolz & Knackmuss, 1993; Yoshida *et al.*, 2004, 2007). Maleylacetate reductases catalyze NADH- or NADPH-dependent reduction, at the carbon–carbon double bond, of maleylacetate or 2-chloromaleylacetate to 3-oxoadipate or of substituted maleylacetates to substituted 3-oxoadipates. The enzymes that use 2-chloromaleylacetate as a substrate initially catalyze the NADH- or NADPH-dependent dechlorination of that compound to maleylacetate, which is subsequently reduced to 3-oxoadipate.

Maleylacetate reductase from *Rhizobium* sp. strain MTP-10005 catalyzes the reduction of maleylacetate to 3-oxoadipate and plays an important role in the catabolic pathway of the resorcinol produced through decarboxylation of 2,6-dihydroxybenzoate (γ-resorcylate; Yoshida *et al.*, 2004, 2007). The polypeptide chain of the enzyme consists of 351 amino-acid residues (Yoshida *et al.*, 2007). The amino-acid sequence of the enzyme is homologous to those of maleylacetate reductases from *Ralstonia eutropha* JMP134 (46%; Seibert *et al.*, 1993), *Pseudomonas* sp. strain B13 (45%; Kasberg *et al.*, 1997) and *Agrobacterium tumefaciens* (90%; Parke, 1995). These homologous enzymes are known to be dimers consisting of two identical subunits. It is also known that they are inhibited by thiol-blocking reagents such as *p*-chloromercuribenzoate and Hg^{2+} (Gaal & Neujahr, 1980; Kaschabek & Reineke, 1993; Seibert *et al.*, 1993), indicating that the cysteine residue is probably necessary for the catalytic activity of maleylacetate reductase. The sequence homology suggests that these properties might be conserved in the present enzyme GraC. GraC may be a dimeric enzyme. The highly conserved cysteine residue Cys242 is found in the C-terminal part (Yoshida *et al.*, 2007). In addition, the sequence motif of the NAD-binding fingerprint, as proposed by Wierenga *et al.* (1986), is found in the region Asp87–Glu123 in the N-terminal part. These findings suggest that the subunit consists of two domains: an N-terminal NAD-binding domain and a C-terminal active-site domain. Here, we report the crystallization and preliminary X-ray diffraction studies of maleylacetate reductase from *Rhizobium* sp. strain MTP-10005. The X-ray crystal structure of the present enzyme is expected to reveal structural features that may be common to maleylacetate reductases from other aerobic microorganisms.

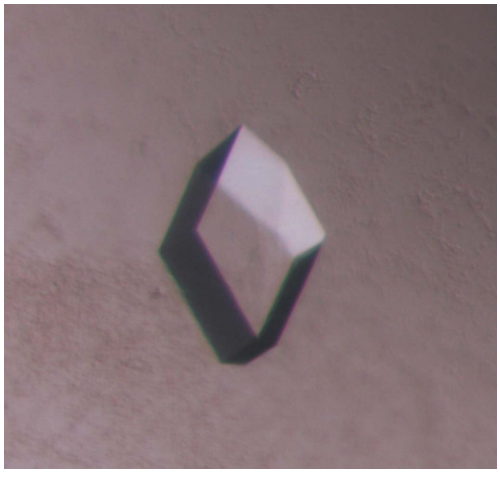

Figure 1
Crystal of maleylacetate reductase (GraC) from *Rhizobium* sp. strain MTP-10005. The dimensions of the crystal were approximately 0.30 × 0.20 × 0.05 mm.

2. Protein expression and purification

Expression of the protein was performed as described previously (Yoshida *et al.*, 2007). The protein was expressed without an inducer because the cloned gene construct does not require an inducer for expression. *Escherichia coli* BL21 Star (DE3) cells harbouring a recombinant plasmid pET14b carrying the *graC* gene were grown in ampicillin-containing LB medium at 310 K. Growth was continued until the optical density reached approximately 0.6 at 600 nm, after which the cultures were placed at 288 K and cultivated for 24 h so as to increase the soluble portion of the protein. After cultivation, the cells were collected by centrifugation (6500*g*, 15 min, 277 K), suspended in 20 m*M* Tris–HCl buffer pH 8.0, washed and repelleted. The washed cells were suspended in buffer *A* (20 m*M* imidazole, 50 m*M* $NaH_2PO_4.H_2O$, 300 m*M* NaCl pH 8.0) and disrupted with an ultrasonic disintegrator. The cell debris was removed by centrifugation (16 000*g*, 15 min, 277 K) to obtain the crude enzyme in the supernatant solution.

Purification of the C-terminally His-tagged protein was performed by chromatography at 277 K and a flow rate of 0.5 ml min^{-1}. The crude enzyme was applied onto an Ni–NTA agarose (Qiagen) column equilibrated with buffer *A* and washed with buffer *B* (50 m*M* imidazole, 50 m*M* $NaH_2PO_4.H_2O$, 300 m*M* NaCl pH 8.0). The adsorbed proteins were eluted with buffer *C* (150 m*M* imidazole, 50 m*M* $NaH_2PO_4.H_2O$, 300 m*M* NaCl pH 8.0). The eluted protein fractions showing a peak in 280 nm absorbance were combined and concentrated by ultrafiltration. The concentrated protein solution was applied onto a PD-10 desalting column equilibrated with 50 m*M* Tris–HCl buffer pH 8.0. The GraC protein was eluted with the same buffer. The purity of the protein was determined by 13%(*w*/*v*) SDS–PAGE analysis. The protein solution was concentrated to 1 mg ml^{-1} and stored at 193 K until the crystallization experiments.

3. Crystallization

Initial crystallization experiments were performed by the hanging-drop vapour-diffusion method using Crystal Screen I (CS I) and Crystal Screen II (CS II) (Hampton Research, California, USA). The protein concentration was adjusted to 10 mg ml^{-1} (CS I Nos. 1–18 and 25–42) or 5 mg ml^{-1} (CS I Nos. 19–24 and 43–50, CS II) in 50 m*M* Tris–HCl buffer pH 8.0. Drops consisting of 1 µl protein solution and 1 µl reservoir solution were equilibrated against 500 µl reservoir solution at 293 K. Small rhombohedron-shaped crystals were obtained after several days with CSI No. 16 and CS II No. 32 and needle crystals appeared within a week with CS I Nos. 6, 9, 15 and 18 and CS II No. 26. The crystallization conditions were optimized based

Table 1
Crystal data and X-ray data-collection statistics.

Values in parentheses are for the highest resolution shell.

X-ray source	PF-AR NW12A
Wavelength (Å)	1.000
Temperature (K)	100
Space group	*C*2
Unit-cell parameters (Å, °)	a = 56.85, b = 121.13, c = 94.09, β = 101.48
Subunits per ASU	2
V_M (Å^3 Da^{-1})	2.18
Solvent content (%)	44.0
Resolution range (Å)	50.0–1.79 (1.85–1.79)
No. of observed reflections	218884
No. of unique reflections	58558 (5814)
Completeness (%)	98.9 (99.9)
R_{merge}† (%)	2.5 (7.9)
Multiplicity	3.8 (3.7)
Average $I/\sigma(I)$	52.7 (14.3)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I(hkl)$ is the intensity of reflection hkl, $\sum_{hkl}$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection hkl.

on those of CS II No. 32 in conjunction with an additive screen (Additive Screen, Hampton Research). The final conditions, after optimization, produced rhombohedron-shaped crystals with approximate dimensions of 0.30 × 0.20 × 0.05 mm at 293 K in 3 d using the sitting-drop vapour-diffusion method (Fig. 1). Drops of 1 µl protein solution at 8 mg ml^{-1} (in 50 m*M* Tris–HCl buffer pH 8.0) and 1 µl reservoir solution were equilibrated against 500 µl reservoir solution consisting of 1.1–1.4 *M* ammonium sulfate, 0.1 *M* sodium chloride, 2%(*w*/*v*) benzamidine–HCl and 0.1 *M* Na HEPES pH 7.5. The drops were microseeded using the original crystals grown in CS II No. 32.

4. X-ray analysis

Diffraction experiments were performed on beamline NW12A, Photon Factory PF-AR, Tsukuba, Japan. A crystal with typical dimensions of 0.30 × 0.20 × 0.05 mm was soaked in a cryoprotectant solution consisting of 1.4 *M* ammonium sulfate and 25%(*v*/*v*) glycerol for less than 1 min and flash-cooled in a nitrogen stream at 100 K. Diffraction data were collected at a wavelength of 1.000 Å using a Quantum 210 CCD detector (ADSC Inc., California, USA) set at a crystal-to-detector distance of 166.2 mm. A data set was collected using 180 frames with a 1.0° oscillation angle and an exposure time of 0.35 s. All diffraction images were processed to 1.79 Å resolution with *HKL*-2000 (Otwinowski & Minor, 1997).

The crystal belongs to space group *C*2, with unit-cell parameters a = 56.85, b = 121.13, c = 94.09 Å, β = 101.48°. The statistics of data collection and processing are summarized in Table 1. Assuming that the crystal contains four dimeric molecules in the unit cell, the V_M value is 2.18 Å^3 Da^{-1}, which is acceptable for a protein crystal (Matthews, 1968). A map of the self-rotation function at 3.0 Å resolution gave a low peak in the κ = 180° plane showing the twofold symmetry relation between the subunits in the molecule. This result may suggest that the structures of the subunits in the dimeric molecule may be slightly different from each other in each domain of the subunit and/or in the relative arrangement between the domains in the subunit. Crystallographic structural studies of GraC from *Rhizobium* sp. strain MTP-10005 have been initiated by searching for a homologous protein of known structure for molecular replacement and by preparing heavy-atom derivatives for isomorphous replacement.

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