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Purification, crystallization and preliminary X-ray diffraction studies on the thermostable catechol 2,3-dioxygenase of *Bacillus stearothermophilus* expressed in *Escherichia coli*

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Abstract

The thermostable catechol 2,3-dioxygenase of *Bacillus stearothermophilus* has been crystallized. The crystal is probably in the space group I_{222} with unit-cell dimensions of a = 70.87, b = 74.60 and c = 133.69 Å. A native data set has been collected with a completeness of 96% at 2.22 Å resolution and an $R_{\rm merge}$ value of 0.091.

1. Introduction

The degradation metabolism of phenolic compounds such as phenol, toluene and xylene in mesophilic bacteria has been extensively studied, especially in *pseudomonads* (Bayly & Barbour, 1984; Nakai *et al.*, 1983). The benzene ring of toluene or related compounds may be cleaved by either the *meta* or *ortho* pathway *via* the intermediate catechol or substituted catechol (Nozaki, 1979). Catechol 2,3-dioxygenase (E.C. 1.13.11.2) catalyzes the extrodiol cleavage of catechol to form 2-hydroxymuconate semialdehyde and plays an important role in metabolism of phenolic compounds (Shu *et al.*, 1995). The gene encoding for the catechol 2,3-dioxygenase in *Pseudomonas* has been cloned and expressed (Kobayashi *et al.*, 1995). The counterpart of the mesophilic enzyme is the thermostable one responsible for the degradation of hazardous phenolic compounds especially in a high-temperature environment by thermophiles. The thermostable enzyme is also an ideal model protein for elucidating the structural basis for protein thermostability owing to its high sensitivity and ease of assaying its activity. The crystal structure of the biphenyl-cleaving extradiol dioxygenase from a PCB-degrading pseudomonad has been reported (Sugiyama *et al.*, 1995; Han *et al.*, 1995; Senda *et al.*, 1996) but the structure of the catechol dioxygenase has not been reported as yet. The research on catechol 2,3-dioxygenase has been hampered by the inability to obtain the complete holoenzyme (Kobayashi *et al.*, 1995).

We have previously cloned the thermostable catechol 2,3-dioxygenase from the thermophile *Bacillus stearothermophilus* FDTP-3 (Dong *et al.*, 1992; He *et al.*, 1995). As reported recently, the enzyme physiologically exists as a homotetramer with an apparent molecular mass of 140 kDa, and the subunit is composed of 327 amino-acid residues. The homology in amino-acid sequence between the mesophilic and thermophilic enzymes is about 27% (He *et al.*, 1995).

2. Purification and crystallization

The enzyme was purified from *Escherichia coli* TG1 cells harboring the overexpression vector of the enzyme gene. Approximately 10 g (wet weight) of cells were disrupted by sonication in 100 ml of buffer *A* containing 50 m*M* Tris–HCl (pH 8.0, 298 K), 20 m*M* NaCl, 5 m*M* EDTA, 10 m*M* β-mercaptoethanol and 5% glycerol. The lysate was incubated at 338 K for 30 min and then cooled on ice. The precipitate containing the cell debris and denatured proteins was removed by centrifugation. The supernatant was applied to a DEAE-52 cellulose column (Whatman) equilibrated with buffer *A*. The column was eluted with a 400 ml linear gradient of 20–500 m*M* NaCl in buffer *A*. The fractions containing the enzyme were collected and dialyzed against buffer *B* containing 50 m*M* Na_2HPO_4–NaH_2PO_4 (pH 6.8, 298 K), 750 m*M* $(NH_4)_2SO_4$, 1 m*M* EDTA and 10 m*M* β-mercaptoethanol, and applied to a phenyl–Sepharose CL-4B column (Pharmacia) equilibrated with buffer *B*. The column was eluted with a linear gradient of 750–0 m*M* $(NH_4)_2SO_4$ in buffer *B*. The resulting purified proteins showed a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The purified enzyme was concentrated to 10–25 mg ml^{-1} in 100 m*M* sodium citrate buffer (pH 5.6, 298 K) for crystallization experiments.

Crystallizations were set up at 277 K by the sparse-matrix approach with the hanging-drop vapor-diffusion method (Jancarik & Kim, 1991). The crystal used for X-ray diffraction study was grown from hanging drop containing 2 µl of a 20 mg ml^{-1} freshly purified enzyme solution and 5 µl of reservoir solution [100 m*M* HEPES (pH 7.5, 298 K), 30%(*w*/*v*) PEG 400 and 0.3 *M* $MgCl_2$ solution].

3. X-ray diffraction studies

A complete native data set was collected from a single crystal of approximate 0.8 × 0.4 × 0.2 mm on a Mar Research 300 mm imaging plate at the Young Scientist Laboratory of Structure Biology, University of Science and Technology of China (USTC) in Hefei. Cu $K\alpha$ X-rays were used at 40 kV and 50 mA. The distance of the crystal to the imaging plate was set to 175 mm for collecting data to a maximum resolution of 2.22 Å. The images were collected as a series of 1° oscillations with a dose exposure of 300 s at room temperature. Data were auto-indexed, integrated and corrected for Lorentz and polarization effects with the program *DENZO* on a Silicon Graphics INDY system. Scaling and merging of data were achieved with the program *SCALEPACK* (Otwinowski, 1993).

The crystals are stable against X-ray exposure. With cell dimensions a = 70.87, b = 74.60 and c = 133.69 Å, the space group is I_{222} or $I2_12_12_1$, based on the observed systematic absences. The space group $I2_12_12_1$ is unlikely because where the twofold axes do not intersect and that would not favor formation of a tetramer. A total of 112 470 observed reflections were merged into 16 912 unique reflections, in the resolution range of 2.22–20 Å. The data were 96% complete to 2.22 Å resolution, with an overall $R_{\rm merge}$ of 0.091. [$R_{\rm merge}$ = $(\sum|I - \langle I\rangle|)/\sum\langle I\rangle$]. According to the molecular weight of

the enzyme subunit and space group of the crystals, it can be inferred that eight molecules are present in unit cell. The V_m value of the crystal is 2.35 $Å^3$ Da^{-1} and the solvent content is about 48% (Matthews, 1968). The crystal structure determination is in progress.

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