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Crystallization and Preliminary X-Ray Diffraction Studies of Expressed *Pseudomonas putida* Catechol 2,3-Dioxygenase[1]

Akiko Kita,* Shin-ichi Kita,*,† Koji Inaka,† Tetsuo Ishida,‡ Kihachiro Horiike,‡ Mitsuhiro Nozaki,‡ and Kunio Miki*,2,3

*Department of Chemistry, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto 606-01; †Research Laboratory of Resources Utilization, Tokyo Institute of Technology, Nagatsuta, Midori-ku, Yokohama 226; and ‡Department of Biochemistry, Shiga University of Medical Science, Seta, Ohtsu, Shiga 520-21

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Crystals of recombinant *Pseudomonas putida* catechol 2,3-dioxygenase, metapyrocatechase, composed of four identical subunits, each with a molecular mass of 35 kDa and one nonheme ferrous iron, have been grown by the vapor diffusion method using sodium citrate as the precipitant. Repeated macroseeding and the addition of ethanol to protein solutions were together effective for obtaining crystals suitable for further crystallographic characterization. The crystals belong to the tetragonal space group $P4_22_12$ with unit-cell dimensions of $a=b=266$ Å, $c=60$ Å. They diffracted beyond 2.5 Å resolution with synchrotron radiation. Assuming that one tetramer $(\alpha\text{-}Fe^{2+})_4$ is contained in an asymmetric unit, the crystal volume per unit molecular mass, V_m, is calculated to be 3.8 Å^3/Da, which corresponds to the solvent content of 67.6%.

Key words: catechol 2,3-dioxygenase, crystallization, metapyrocatechase, non-heme iron dioxygenase, X-ray crystallography.

Dioxygenase containing nonheme iron as a sole cofactor plays a key role in the oxidative biodegradation of a large number of aromatic compounds by catalyzing the cleavage of aromatic rings. Catechol 2,3-dioxygenase [catechol: oxygen 2,3-oxidoreductase (decyclizing), EC 1.13.11.2, metapyrocatechase] is an extradiol-type dioxygenase that catalyzes the conversion of catechol to 2-hydroxymuconate semialdehyde with the insertion of two atoms of dioxygen (Fig. 1) (*1*). Catechol 2,3-dioxygenase from *Pseudomonas putida* mt-2 (ATCC 23973) is composed of four identical subunits with one ferrous ion bound to each subunit: the enzyme is designated as $(\alpha\text{-}Fe^{2+})_4$ (*2, 3*). The enzyme subunit of molecular mass 35 kDa consists of 307 amino acid residues. The enzyme is easily inactivated by various oxidizing agents, such as air. The inactivation appears to be due to oxidation of ferrous ions to the ferric form (*4, 5*). The presence of an organic solvent such as acetone or ethanol protects the enzyme from inactivation (*5*). Various phenol derivatives, for example, *o*-nitrophenol, inhibit the enzyme activity (*6*).

Elucidation of the catalytic mechanism of this enzyme requires knowledge of the active site structure at an atomic resolution, which can be obtained only by determining the three-dimensional structure. We have reported the expression of *Pseudomonas putida* catechol 2,3-dioxygenase at a high level by *Escherichia coli* and detailed characterization of the recombinant enzyme (*6*). Here we describe the successful crystallization and preliminary crystallographic characterization of the enzyme overexpressed heterologously. The crystal structures of dioxygenases have been reported for protocatechuate 3,4-dioxygenase (*7, 8*), arachidonic acid 15-lipoxygenase (*9*), and 2,3-dihydroxybiphenyl dioxygenase (*10-12*). The structure of catechol 2,3-dioxygenase will give important information on the catalytic mechanism of a series of dioxygenases.

EXPERIMENTAL PROCEDURES

Crystallization—Expression and purification of the recombinant catechol 2,3-dioxygenase were done as described previously (*6*). Protein solutions were dialyzed against 50 mM Tris-acetate containing 10% (v/v) acetone (pH 8.0). After dialysis the enzyme solutions were concentrated to 70-80 mg/ml by using a microconcentrator (Centricon 100, Amicon). The sitting-drop vapor diffusion method was employed for crystallization. Droplets (6 μl) of the protein solutions were pipetted onto glass slides, and vapor-equilibrated against 500 μl of reservoir solutions containing higher concentrations of precipitants than the droplets. Ammonium sulfate, lithium sulfate, potassium phosphate, ammonium citrate, sodium citrate, magnesium chloride, polyethylene glycol, 2-methyl-2,4-pentanediol, acetone, and ethanol were used as precipitating agents. All crystallizations were carried out in a cold room controlled at 4°C.

X-Ray Diffraction Study—The crystals were mounted in

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[2] To whom correspondence should be addressed. e-mail: miki@kuchem.kyoto-u.ac.jp

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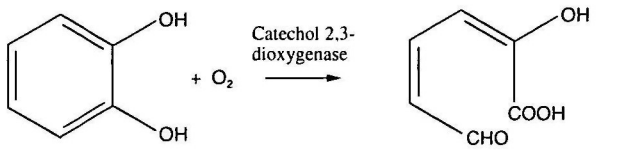
a glass capillary with a trace amount of the mother liquor. For crystallographic characterization, oscillation photographs were taken on a DIP100s imaging-plate camera system (Mac Science) using with Cu-$K\alpha$ radiation generated on the M18X rotating anode generator operated at 50 kV and 90 mA with a fine focus filament. The crystal-to-film distance was set to 100 mm. The unit-cell dimensions were refined on oscillation photographs by the program *DENZO* (*13*).

For both crystallographic characterization and intensity data collection, oscillation and Weissenberg photographs were taken on a 200×400 mm imaging plates by a screenless Weissenberg camera (*14*) with a 0.1-mm aperture collimator and a cylindrical cassette of 430 mm radius using 2.5 GeV synchrotron radiation at the BL-6A beam line of the Photon Factory, the National Laboratory for High Energy Physics. The X-ray beam was monochromatized to 1.00 Å by an Si(111) monochromator system. The imaging-plates were digitized at 100-μm intervals on a Fujix BA100 read-out system (Fuji Photo Film) (*15*). Intensity data were evaluated using the *WEIS* program (*16*) on a FACOM M780/10 computer. After the intensities of individual plates had been scaled and combined within each crystal, data processing was carried out by *PROTEIN* program system (*17*) on a VAXstation 4000/90 computer.

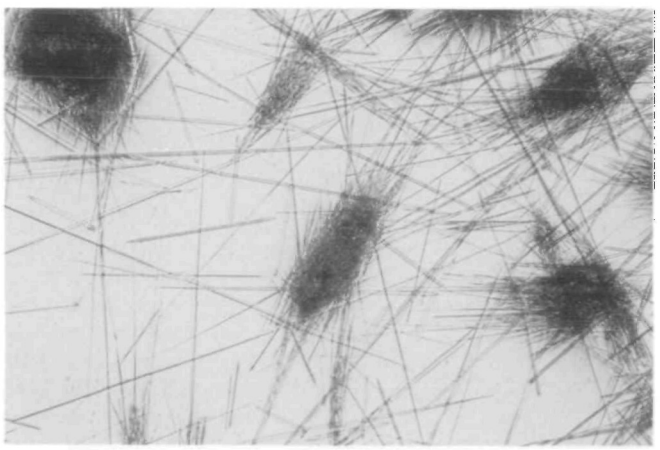
RESULTS AND DISCUSSION

Crystals of catechol 2,3-dioxygenase were obtained after

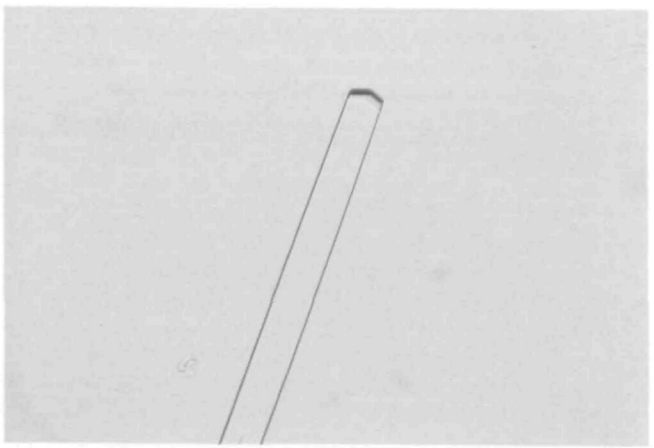


Catechol 2-Hydroxymuconate semialdehyde

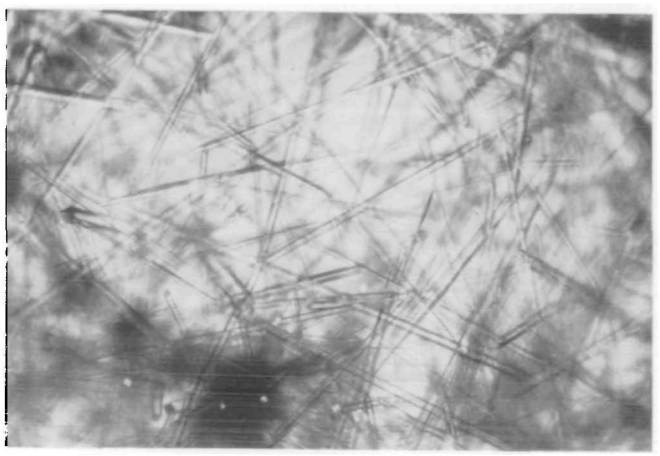
Fig. 1. **The extradiol-type dioxygenase reaction catalyzed by catechol 2,3-dioxygenase.**



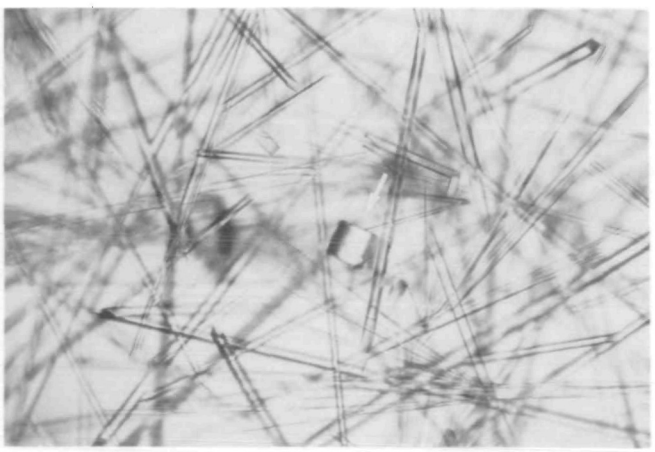
(a)



(c)



(b)



(d)

Fig. 2. **Crystals of *Pseudomonas putida* catechol 2,3-dioxygenase.** (a)Needle crystals obtained from ammonium sulfate solutions. Protein solutions (20 mg/ml) with 15% (w/v) ammonium sulfate were vapor-equilibrated against 18–24% (w/v) precipitant solutions at 4°C. (b) Crystals obtained from sodium citrate solutions. Protein solutions (20 mg/ml) with 15% (w/v) sodium citrate were vapor-equilibrated against 26–30% (w/v) solutions at 4°C. (c) Crystal obtained by addition of ethanol to the protein solutions and used for as a seed in the stepwise macroseeding procedure. (d) Crystals obtained after the stepwise macroseeding procedure in sodium citrate solutions. The well-grown seed crystal (white arrow) is located so that its long edge (c^* axis) is perpendicular to the surface of the drop of protein solutions.

one week at 4°C in 10-20% (w/v) solutions of the inorganic precipitants, except for magnesium chloride, with protein concentrations of 15-40 mg/ml. All crystals except for those obtained from sodium citrate were needle-like and too thin to handle (Fig. 2a). The crystals obtained from sodium citrate were slightly but significantly thicker prisms, but they formed aggregates in four or five days (Fig. 2b).

The best crystals were obtained from sodium citrate solution in the following way. Protein solutions in 15% (w/v) sodium citrate (20 mg/ml) were vapor-equilibrated at 4°C against outer solutions containing 26-30% (w/v) of the precipitant. Addition of ethanol to both inner and outer solutions [final concentrations of 3% (v/v)] prevented the aggregation of crystals. However, the prismatic crystals grown by the conventional macroseeding procedure were still too small for X-ray diffraction work, their average dimensions being $0.02\times0.02\times0.5$ mm (Fig. 2c). Accordingly, the following stepwise macroseeding method was employed. First, a thin prismatic crystal was placed in a solution containing a lower salt concentration than that used for the initial crystallization, and this induced the formation of many cracks in the crystal, most of which ran perpendicular to the long axis. Second, the crystal was cut into several pieces along the cracks. Third, a piece of crystal was placed into the protein solution as a seed with its long edge standing vertically in the drop. By repeating this macroseeding procedure several times, the seeded crystal was grown to a maximal size of $0.1\times0.1\times1.0$ mm (Fig. 2d).

Figure 3 shows an image pattern (the central part) of the oscillation photograph taken with synchrotron radiation. The Laue symmetry and the systematic absences of reflections indicated that the crystals belong to the tetragonal space group $P4_22_12$. The unit-cell dimensions were determined as $a=b=266$ Å and $c=60$ Å by the refinement of reflections recorded on 1° rotated oscillation photographs taken on a DIP100s imaging-plate camera system. Assuming one or two tetrameric molecules of catechol 2,3-dioxygenase per asymmetric unit, the crystal volume per unit molecular mass, V_m, is calculated to be 3.8 or 1.9 Å^3/Da, respectively (*18*).

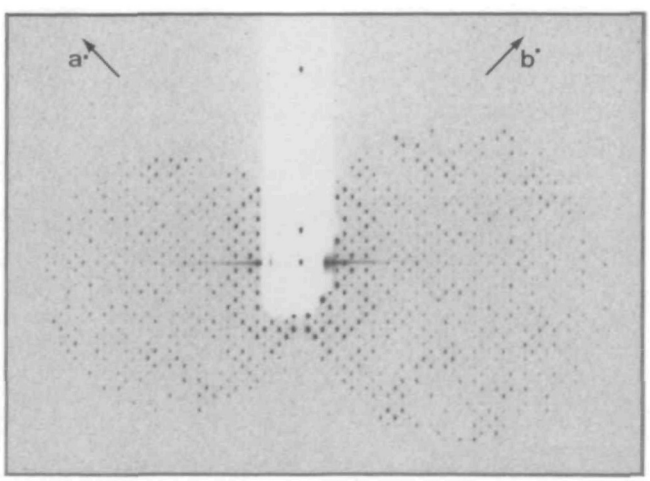


Fig. 3. **Oscillation photograph taken with rotation of the a^*+b^* axes on the screenless Weissenberg camera using synchrotron radiation (wavelength 1.00 Å).** Oscillation range is 6° and the crystal-to-film distance is 429.7 mm.

A full set of the native intensity data was also collected with synchrotron radiation using one crystal. A crystal with approximate dimensions of $0.1\times0.1\times1.0$ mm was mounted in a capillary such that it could be rotated about the [001] axis. The total oscillation range of 48.5° was covered by 11 serial Weissenberg photographs, in which the oscillation range of 4.5°C was employed. The exposure time was 60 s per degree. Diffraction spots from the native crystals were recorded beyond 2.5 Å resolution, indicating that they are suitable for X-ray crystal structure determination at an atomic resolution. After averaging of symmetry-equivalent reflections up to 2.5 Å resolution, a total of 45,370 unique reflections was obtained, which corresponds to 60% of the number of theoretically measurable reflections. The merging R factor defined by $R_{merge}=\Sigma\Sigma|I_{hj}\langle I\rangle_h|/\Sigma_h\Sigma_{hj}I_{hj}$ is 0.072 for 116,076 measurements, where $\langle I\rangle_h$ is the mean intensity of a reflection h, and I_{hj} is the jth measurement of reflection h.

It can be concluded that repeated macroseeding and the addition of ethanol to protein solutions were together effective in improving such crystal habits as the assembly of thin needles in order to obtain crystals suitable for further crystallographic work. A search for heavy atom derivatives is in progress using the crystals obtained in order to establish the three-dimensional structure.

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