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# 4-Chlorocatechol 1,2-dioxygenase from the chlorophenol-utilizing Gram-positive *Rhodococcus opacus* 1CP: crystallization and preliminary crystallographic analysis

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4-Chlorocatechol 1,2-dioxygenase (4-ClC1,2DO) from the Gram-positive bacterium *Rhodococcus opacus (erythropolis)* 1CP, an enzyme involved in the aerobic biodegradation of chloroaromatic compounds, has been crystallized. 4-ClC1,2DO, which specifically catalyzes the intradiol cleavage of 4-substituted catechols, which are intermediates in the degradation of a variety of aromatic pollutants, to the corresponding maleylacetates, has recently been purified to homogeneity. The enzyme is an homodimer composed of two identical subunits in an $\alpha_2$-type quaternary structure; it has a molecular weight of about 29 kDa per monomer and contains one $Fe^{III}$ and one $Mn^{II}$ ion per homodimer. Hexagonal crystals grown in 1.6 *M* ammonium sulfate, 0.1 *M* sodium chloride, 100 m*M* Tris–HCl pH 9.0, 5–15% glycerol were successfully frozen under liquid nitrogen, adding 30% glycerol to the mother-liquor solution as a cryoprotectant. A complete data set was collected at 2.8 Å resolution using the EMBL beamline BW7A at the DORIS storage ring, DESY, Hamburg, Germany with a MAR CCD detector and a wavelength of 1.01 Å. The crystals belong to the primitive hexagonal space group $P6_322$, with unit-cell parameters $a$ = 90.4, $c$ = 307.5 Å. This is the first intradiol dioxygenase which specifically catalyzes the cleavage of chlorocatechols in Gram-positive bacteria to give diffraction-quality crystals.

## 1. Introduction

The aerobic metabolism of chloroaromatics, a class of compounds highly recalcitrant to biodegradation, generally occurs through two different pathways depending on the number of chlorine substituents on the aromatic ring (Chaudhry & Chapalamadugu, 1991; Reineke & Knackmuss, 1988). Those compounds having one or two chlorines are usually converted to chlorocatechols and then catabolized through the modified *ortho*-cleavage pathway, which is specific for the assimilation of chloroaromatics and was first identified in Gram-negative strains (Bollag *et al.*, 1968; Tiedje *et al.*, 1969; Duxbury *et al.*, 1970). On the other hand, aromatic compounds containing more than two chlorine substituents are converted to hydroxyquinol or chlorohydroxyquinol and then cleaved by specific intradiol dioxygenases (Apajalahti & Salkinoja-Salonen, 1987; Joshi & Gold, 1993; Kozyreva *et al.*, 1993; Li *et al.*, 1991; Sangodkar *et al.*, 1988; Sze & Dagley, 1984; Travkin *et al.*, 1997).

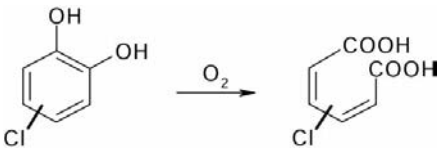
The only known exception to this scheme is the microorganism *Pseudomonas chlororaphis* RW71, which is able to mineralize 1,2,3,4-tetrachlorobenzene through the formation and subsequent cleavage of 3,4,5,6-chlorocatechol catalyzed by a specialized chlorocatechol 1,2-dioxygenase (Potrawfke *et al.*, 1998, 2001).

An unusual extradiol catechol 2,3-dioxygenase that converts 3-chlorocatechol and 3-methylcatechol has been recently purified from *P. putida* GJ31. This is the only known organism to use both chloroaromatics and methylaromatics for growth *via* a pathway involving extradiol cleavage (Kaschabek *et al.*, 1998).

The capability of Gram-positive bacteria to use chloroaromatic compounds as sole carbon and energy sources has only recently been observed (Golovlev & Eroshina, 1982; Janke *et al.*, 1986; Häggblom *et al.*, 1989; Ihn *et al.*, 1989), as the majority of the representatives of the nocardiaform genus *Rhodococcus* do not show the presence of an appropriate modified *ortho*-cleavage pathway (Janke *et al.*, 1989).

Chlorocatechol 1,2-dioxygenases are key enzymes of this modified *ortho*-cleavage pathway and generally show high substrate specificities.

The chlorocatechol 1,2-dioxygenases (ClC1,2DO) catalyze the intradiol cleavage of chlorocatechols to chloro-*cis*,*cis*-muconates.




Several ClC1,2DOs have been purified from a variety of microorganisms, in particular from Gram-negative [*Arthrobacter*, *Pseudomonas* sp. B13, *Pseudomonas* sp. P51, *P. putida* AC866, *P. putida* EST4021, *Ralstonia eutropha (Alcaligenes eutrophus)* JMP134] and more recently also from Gram-positive [*Rhodococcus opacus (erythropolis)* 1CP] bacteria, but little is known about these enzymes or the factors discriminating for substrate specificity of this novel group of intradiol dioxygenases (Dorn & Knackmuss, 1978*a*,*b*; Reineke & Knackmuss, 1988; Ngai & Ornston, 1988; Pieper *et al.*, 1988; Broderick & O'Halloran, 1991; Solyanikova *et al.*, 1992; Hinteregger *et al.*, 1992; Bhat *et al.*, 1993; Miguez *et al.*, 1993; Maltseva *et al.*, 1994).

4-ClC1,2DO from *R. opacus (erythropolis)* 1CP is an homodimer with a molecular weight of about 29 kDa per monomer, containing one $Fe^{III}$ ion and one $Mn^{II}$ ion (Maltseva *et al.*, 1994; Eulberg *et al.*, 1998). A recent X-ray absorption spectroscopy study shows that in the native enzyme the iron is pentacoordinate with average Fe—*L* distance of 1.96 Å and that histidines are present in the metal-coordination sphere (Briganti *et al.*, 1998). The study also shows that pentacoordination is maintained in the enzyme–substrate complex and that the first metal-coordination sphere is not affected by substrate binding.

To date, the X-ray structures of only a few intradiol dioxygenases, the protocatechuate 3,4-dioxygenases (3,4PCDO) from *P. aeruginosa* and *Acinetobacter* sp. ADP1, their adducts with substrates and inhibitors and the catechol 1,2-dioxygenase from *Acinetobacter* sp. ADP1, have been determined (Elgren *et al.*, 1997; Ohlendorf *et al.*, 1988, 1994; Orville *et al.*, 1997; Vetting & Ohlendorf, 2000; Vetting *et al.*, 2000). Diffraction-quality crystals have also been obtained and X-ray diffraction studies are in progress for a few intradiol dioxygenases with different substrate specificities such as the catechol 1,2-dioxygenase isoenzymes from *P. putida (arvilla)* C-1, the hydroxyquinol 1,2-dioxygenase from *Nocardioides simplex* 3E and the 3,4PCDOs from *P. cepacia* and *Brevibacterium fuscum* (Benvenuti *et al.*, 1999; Earhart, Hall *et al.*, 1994; Earhart, Radhakrishnan *et al.*, 1994; Ludwig *et al.*, 1984).

**Figure 1**
Crystals of 4-chlorocatechol 1,2-dioxygenase from *R. opacus* 1CP

In this paper, we report the crystallization of 4-chlorocatechol 1,2-dioxygenase from the Gram-positive bacterium *R. opacus (erythropolis)* 1CP. X-ray data have been collected from native enzyme crystals, providing a complete data set at 2.8 Å. This is the first dioxygenase that catalyzes the degradation of chlorocatechols to be crystallized and shown to produce diffraction-quality crystals. Determination of the three-dimensional structure of this enzyme will provide details on the conformation of the active site and will allow further improvement of the knowledge of the catalytic mechanism of such class of dioxygenases.

## 2. Crystallization

4-Chlorocatechol 1,2-dioxygenase from *R. opacus (erythropolis)* 1CP was purified as previously reported (Maltseva *et al.*, 1994).

Initial crystallization trials were performed with Hampton Research Crystal Screen and Crystal Screen 2 using the sitting-drop vapour-diffusion method. 1 µl of a 20 mg ml$^{-1}$ protein solution in 20 m*M* Tris–$SO_4$ pH 7.2 with 1 µl reservoir solution was equilibrated against 500 µl of precipitant solution. Certain conditions (4 and 29 of Hampton Research Crystal Screen, and 23, 32, 41 and 42 of Hampton Research Crystal Screen 2) produced rosettes or hexagonal crystals with growth defects. The best results were obtained with condition 32 (1.6 *M* ammonium sulfate, 0.1 *M* sodium chloride, 100 m*M* HEPES pH 7.5) and this was used for optimization. The concentrations of the protein and salts and the pH were systematically varied and trials were also performed in order to attempt to grow larger and regularly shaped crystals by using additives, oils and seeding.

The optimized crystallization buffer contains 1.6 *M* ammonium sulfate, 0.1 *M* sodium chloride, 100 m*M* Tris–HCl pH 9.0 and 5–15% glycerol.

Crystals of a different morphology were obtained by adding 3% 1,2,3-heptanetriol to condition 32 of Hampton Research Crystal Screen 2. In these drops, many small crystals of an elongated prismatic shape grew; any attempts to increase their dimensions failed. However, the largest obtained crystals of this form showed no diffraction.

**Table 1**
Crystal parameters and data-collection statistics.

Values in parentheses correspond to the highest resolution shell, 2.85–2.80 Å.

| | |
|---|---|
| Space group | $P6_322$ |
| Unit-cell parameters (Å) | |
| *a* | 90.4 |
| *c* | 307.5 |
| Unit-cell volume (Å$^3$) | 2177246 |
| $V_M$ (Å$^3$ Da$^{-1}$) | 3.13 (61% solvent) |
| Asymmetric unit content | 1 molecule (2 subunits) |
| Limiting resolution (Å) | 2.8 |
| Total reflections measured | 109132 |
| No. of unique reflections | 17339 |
| $R_{sym}$† (%) | 8.3 (23.4) |
| Completeness (%) | 89.9 (70.4) |
| $\langle I/\sigma(I)\rangle$ | 16.6 (3.1) |
| Redundancy | 6.3 |

† $R_{sym} = \sum|I_i - \langle I\rangle|/\sum\langle I\rangle$, where $I_i$ is an individual intensity measurement and $\langle I\rangle$ is the average intensity for this reflection with summation over all data.

## 3. Data collection

The hexagonal crystals (see Fig. 1) were successfully frozen under liquid nitrogen after adding 30% glycerol to the mother-liquor solution as cryoprotectant.

Crystals of this form were tested using a rotating-anode source coupled with a 1K CCD detector from Bruker and showed diffraction to ∼3.5 Å at best.

A complete data set at 100 K was collected at the EMBL beamline BW7A at the DORIS storage ring, DESY, Hamburg, Germany. Data were collected at 2.8 Å using a MAR CCD detector and a wavelength of 1.01 Å. The crystals belong to the primitive hexagonal space group $P6_322$, with unit-cell parameters $a$ = 90.4, $c$ = 307.5 Å. Assuming one molecule per asymmetric unit, the solvent content is about 61% of the unit cell ($V_M$ = 3.13 Å$^3$ Da$^{-1}$; Matthews, 1968).

Data processing with *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997) gave 17 339 unique reflections, an $R_{sym}$ of 8.3% and an overall completeness of 89.9%. A summary of data collection and processing is given in Table 1.

All molecular-replacement attempts using the coordinates of known intradiol dioxygenases structures as a models have so far failed to provide a solution for 4-chloro-

catechol 1,2-dioxygenase (Elgren *et al.*, 1997; Ohlendorf *et al.*, 1988, 1994; Orville *et al.*, 1997; Vetting & Ohlendorf, 2000; Vetting *et al.*, 2000). Our efforts are currently being directed towards a search for heavy-atom derivatives and the solution of the structure using multiple isomorphous replacement.

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