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Preliminary crystallographic analysis of 3-chlorocatechol 1,2-dioxygenase of a new modified *ortho*-pathway from the Gram-positive *Rhodococcus opacus* 1CP grown on 2-chlorophenol

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3-Chlorocatechol 1,2-dioxygenase (3-ClC1,2DO), a key enzyme of a new modified *ortho*-pathway, was isolated from a variant of the Gram-positive bacterium *Rhodococcus opacus* 1CP utilizing 2-chlorophenol as the sole energy and carbon source *via* a 3-chlorocatechol branch of a modified *ortho*-pathway. 3-ClC1,2DO catalyzes the intradiol cleavage of 3-chlorocatechol. The enzyme contains Fe^{III} ions essential to the catalytic activity; it is a homodimer with a molecular weight of about 58 kDa composed of two identical subunits in an $(\alpha Fe)_2$-type quaternary structure. Its physicochemical properties are intermediate between those of the pyrocatechase from the ordinary pathway and those of the chloro-pyrocatechase from the modified pathway described previously for this strain. 3-ClC1,2DO was crystallized using the sitting-drop vapour-diffusion method. After 2 d, prismatic crystals grew in 15% PEG 8000, 0.3 *M* magnesium acetate, 100 m*M* HEPES pH 7.5, 5% glycerol. X-ray diffraction data were collected from a frozen crystal to a maximum resolution of 2.0 Å using 25% PEG 400 as cryoprotectant at the Elettra synchrotron source, Trieste, Italy, at a wavelength of 1.01 Å using a MAR CCD detector. The crystals belong to space group *P*1, with unit-cell parameters $a = 83.18$, $b = 86.61$, $c = 93.44$ Å. Assuming a reasonable range for V_M, the asymmetric unit could contain from three to five $(\alpha Fe^{III})_2$ dimers. A peak present in the $\kappa = 180^\circ$ and $\kappa = 90^\circ$ sections is consistent with a fourfold axis and four dimers in the asymmetric unit. Comparison of the crystal structure of this enzyme with that of the 4-chlorocatechol 1,2-dioxygenase recently crystallized from the same bacterium (Ferraroni *et al.*, 2002) may reveal important details of the influence of the active-site conformation and the amino-acid substitutions involved in substrate selectivity.

1. Introduction

The bacterium *Rhodococcus opacus* 1CP can utilize or transform a number of toxic halogenated phenols that are usually recalcitrant to degradation (Gorlatov *et al.*, 1989; Bondar *et al.*, 1998).

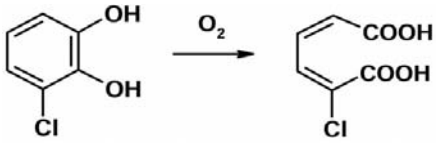
This strain was initially isolated from an enriched culture on 2,4-dichlorophenol and was shown to be able to degrade 2,4-dichlorophenol as well as 4-chlorophenol *via* a modified *ortho*-cleavage pathway *via* the corresponding chlorocatechols, chloromuconates, (chloro)dienelactones and maleylacetates as common intermediates (Bollag *et al.*, 1968; Tiedje *et al.*, 1969; Duxbury *et al.*, 1970; Chaudhry & Chapalamadugu, 1991; Reineke & Knackmuss, 1988; Gorlatov *et al.*, 1989).

Although cells of *R. opacus* 1CP grown on 4-chlorophenol were not able to convert any intermediates of 2-chlorophenol degradation, after a long adaptation a variant of *R. opacus* 1CP grown on 2-chlorophenol as the sole carbon and energy source was obtained and degradation of 2-chlorophenol was shown to proceed *via* a different and new modified *ortho*-cleavage pathway (Moisseeva *et al.*, 1999).

Consequently, degradation of 4-chlorophenol by this strain proceeds *via* the known modified *ortho*-cleavage pathway, whereas degradation of 2-chlorophenol occurs *via* a new modified *ortho*-cleavage pathway composed of enzymes with different substrate specificities.

Recent studies on the biodegradative potential of these two pathways showed significant differences at both the genetic and the biochemical levels and in particular revealed the presence of a new chloromuconolactone isomerase enzyme for the differential biodegradation of the catabolites of 2- and 4-chlorophenol (Maltseva *et al.*, 1994; Solyanikova *et al.*, 1995; Eulberg *et al.*, 1998; Moisseeva *et al.*, 2001, 2003).

One of the key reactions in the first biodegradation steps of haloaromatic compounds is the cleavage of the aromatic ring to chloro-*cis*,*cis*-muconates catalyzed by chlorocatechol dioxygenases.



Studies of the two chlorocatechol 1,2-dioxygenases involved in the degradation pathways for 4-chlorophenol (4-chlorocatechol 1,2-dioxygenase) and 2-chlorophenol (3-chlorocatechol 1,2-dioxygenase) from *R. opacus* 1CP ascertained that these enzymes differ significantly in their catalytic properties (Maltseva *et al.*, 1994; Moisseeva *et al.*, 2001).

3-ClC1,2DO is able to split a restricted number of chloro-substituted catechols, but is characterized by relatively similar values of the specificity constant for all of its substrates (catechol, 3- and 4-methylcatechol, 3- and 4-chlorocatechol). Contrastingly, the 4-chlorocatechol 1,2-dioxygenase from the same strain is able to split various substituted catechols, but *para*-substituted catechols were better substrates for this enzyme than *meta*-substituted catechols (Maltseva *et al.*, 1994).

Furthermore, the sequence homology between 4-chlorocatechol 1,2-dioxygenase and 3-ClC1,2DO from *R. opacus* 1CP is about 42% (unpublished observations).

3-ClC1,2DO from *R. opacus (erythropolis)* 1CP is a homodimer with a molecular weight of about 29 kDa per monomer, containing one Fe^{III} ion per monomer (Moisseeva *et al.*, 2001).

Until now, the X-ray structures of only a few intradiol dioxygenases, the catechol 1,2-dioxygenase from *Acinetobacter* sp. ADP1 and the protocatechuate 3,4-dioxygenases (3,4PCDOs) from *Pseudomonas aeruginosa* and *Acinetobacter* sp. ADP1, have been determined; these show large differences in oligomeric structures and show sequence homologies with the present 3-ClC1,2DO of 33, 35 and 30%, respectively (Elgren *et al.*, 1997; Ohlendorf *et al.*, 1988, 1994; Orville *et al.*, 1997; Vetting & Ohlendorf, 2000; Vetting *et al.*, 2000). These data, together with the information obtained through XAS experiments, are not sufficient to establish the roles of the active-site residues in substrate selectivity (Briganti *et al.*, 1998). X-ray diffraction studies are in progress for a small number of intradiol dioxygenases with different substrate specificities, such as the catechol 1,2-dioxygenase isoenzymes from *Pseudomonas putida (arvilla)* C-1, the hydroxyquinol 1,2-dioxygenase from *Nocardioides simplex* 3E and the 3,4PCDOs from *Pseudomonas cepacia* and *Brevibacterium fuscum* (Benvenuti *et al.*, 1999; Earhart, Hall *et al.*, 1994; Earhart, Radhakrishnan *et al.*, 1994; Ludwig *et al.*, 1984).

In the present paper, we report the crystallization of 3-ClC1,2DO from the Gram-positive bacterium *R. opacus* 1CP grown on 2-chlorophenol. X-ray data have been collected from native enzyme crystals, providing a complete data set at 2.0 Å. The related 4-chlorocatechol 1,2-dioxygenase from the same *R. opacus* 1CP strain has recently been crystallized in our laboratory (Ferraroni *et al.*, 2002).

Determination of the three-dimensional structures of both of these enzymes with different substrate specificities will provide details on the influence of the active-site conformation and the amino-acid substitutions involved in substrate selectivity. It will also improve our knowledge of the catalytic mechanism of this class of dioxygenases.

2. Crystallization

3-ClC1,2DO from *R. opacus (erythropolis)* 1CP was purified as previously reported (Moisseeva *et al.*, 2001).

Initial crystallization trials were performed using the Hampton Research Crystal Screens I and II with the sitting-drop vapour-diffusion method. 1 µl of a 15 mg ml^{-1} protein solution in 20 m*M* Tris–SO_4 pH 7.2 was mixed with an equal amount of reservoir solution and equilibrated against 50 µl of precipitant solution in a 96-well Cryschem Plate (Hampton Research Cat. No. HR3-160).

Certain conditions (Nos. 18, 28 and 39 in Crystal Screen I and No. 26 in Crystal Screen 2) produced crystals. The best results were obtained with condition No. 18 from Crystal Screen I [0.2 *M* $Mg(CH_3CO_2)_2$, 0.1 *M* cacodylate pH 6.5, 20% PEG 8000] and this was used for optimization. The concentrations of the protein and the precipitants and the pH were systematically varied and trials were also made to attempt to grow larger regular-shaped crystals using additives, oils and agarose gel.

The optimized crystallization buffer contains 0.3 *M* $Mg(CH_3CO_2)_2$, 0.1 *M* HEPES pH 7.5, 15% PEG 8000, 5% glycerol. Single crystals from this condition usually appear after 2 d at 295 K, reaching maximum dimensions of 0.2 × 0.5 × 0.5 mm.

3. Data collection

Crystals of 3-ClC1,2DO from *R. opacus* 1CP (see Fig. 1) were successfully frozen under liquid nitrogen, adding 25% of PEG 400 to the mother-liquor solution as a cryoprotectant.

Figure 1
Crystals of 3-ClC1,2DO from *R. opacus* 1CP.

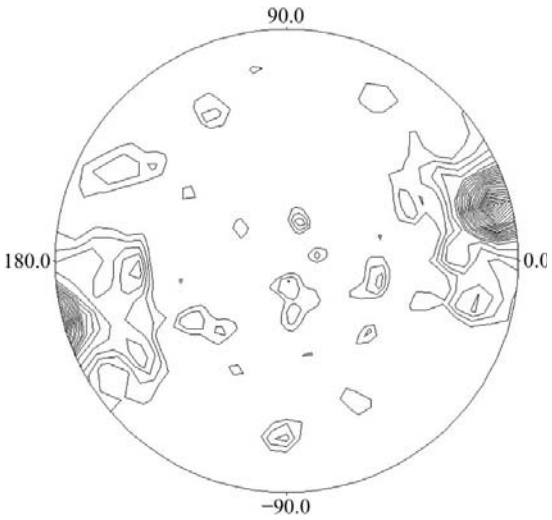


Figure 2
The $\kappa = 90^\circ$ section of the self-rotation function. The self-rotation function was calculated using the program *POLARRFN* from the *CCP*4 package, using a 20 Å radius of integration and data in the resolution range 7–5 Å. A fourfold axis can clearly be seen at $(\omega, \varphi) = (86, 15^\circ)$.

Table 1
Crystal parameters and data-collection statistics for 3-ClC1,2DO from *R. opacus* 1CP.

Values in parentheses are for the highest resolution shell.

Beamline	Elettra (Trieste, Italy)
Space group	*P*1
Unit-cell parameters	
a (Å)	83.18
b (Å)	86.61
c (Å)	93.44
α (°)	85.4
β (°)	66.5
γ (°)	76.9
Unit-cell volume (Å^3)	601225
V_M (Å^3 Da^{-1})	2.59 (52% solvent)
Asymmetric unit content	4 dimers
Limiting resolution (Å)	2.0
Total reflections measured	753103
No. of unique reflections	152395
R_{sym}† (%)	5.8 (12.9)
Completeness (%)	97.6 (91.8)
$I/\sigma(I)$	23.6 (4.1)
Redundancy	4.9

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

Diffraction data were collected at a temperature of 100 K at the X-ray diffraction beamline of the Elettra synchrotron (Trieste, Italy) using a wavelength of 1.01 Å. Data were collected using a MAR CCD detector to a maximum resolution of 2.0 Å. The crystals belong to the primitive triclinic space group *P*1, with unit-cell parameters a = 83.18, b = 86.61, c = 93.44 Å, α = 85.4, β = 66.5, γ = 76.9°. Assuming a reasonable range for V_M, the asymmetric unit could contain from three to five $(\alpha Fe^{III})_2$ dimers (molecular mass of 29 kDa per monomer). To determine which value is correct, a self-rotation function was calculated using the program *POLARRFN* from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994). The peaks present in the κ = 180° and κ = 90° sections are consistent with a fourfold axis and four dimers in the asymmetric unit (Fig. 2).

Data processing with *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997) gave 152 395 unique reflections, an R_{sym} of 0.058 and an overall completeness of 97.6%. A summary of data collection and processing is given in Table 1.

All molecular-replacement attempts using coordinates from known intradiol dioxygenase structures (Elgren *et al.*, 1997; Ohlendorf *et al.*, 1988, 1994; Orville *et al.*, 1997 ; Vetting & Ohlendorf, 2000; Vetting *et al.*, 2000) as a model have so far failed to provide a solution for 3-chlorocatechol dioxygenase. MAD/SAD phasing on the Fe^{III} edge and molecular replacement, using other coordinates from a related intradiol dioxygenase currently at an early stage of refinement in our laboratory, will be explored in order to solve the enzyme crystal structure.

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