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Preliminary crystallographic analysis of salicylate 1,2-dioxygenase from *Pseudaminobacter salicylatoxidans*

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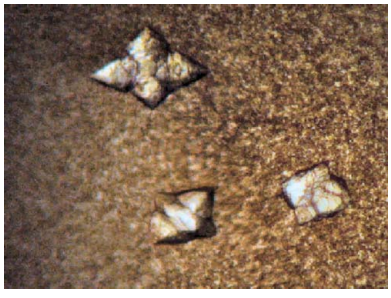
Salicylate 1,2-dioxygenase, a new ring-fission dioxygenase from the naphthalenesulfonate-degrading strain *Pseudaminobacter salicylatoxidans* which oxidizes salicylate to 2-oxohepta-3,5-dienedioic acid by a novel ring-fission mechanism, has been crystallized. Diffraction-quality crystals of salicylate 1,2-dioxygenase were obtained using the sitting-drop vapour-diffusion method at 277 K from a solution containing 10%(*w*/*v*) ethanol, 6%(*w*/*v*) PEG 400, 0.1 *M* sodium acetate pH 4.6. Crystals belong to the primitive tetragonal space group $P4_32_12$ or $P4_12_12$, with unit-cell parameters a = 133.3, c = 191.51 Å. A complete data set at 100 K extending to a maximum resolution of 2.9 Å was collected at a wavelength of 0.8423 Å. Molecular replacement using the coordinates of known extradiol dioxygenases structures as a model has so far failed to provide a solution for salicylate 1,2-dioxygenase. Attempts are currently being made to solve the structure of the enzyme by MAD experiments using the anomalous signal of the catalytic Fe^{II} ions. The salicylate 1,2-dioxygenase structural model will assist in the elucidation of the catalytic mechanism of this ring-fission dioxygenase from *P. salicylatoxidans*, which differs markedly from the known gentisate 1,2-dioxygenases or 1-hydroxy-2-naphthoate dioxygenases because of its unique ability to oxidatively cleave salicylate, gentisate and 1-hydroxy-2-naphthoate with high catalytic efficiency.

1. Introduction

The oxygenolytic cleavage of the aromatic nucleus by bacteria as a general rule demands the presence of two hydroxyl groups attached to the aromatic ring (Bugg, 2003; Costas *et al.*, 2004). Only a few instances have been described in which monohydroxylated aromatic compounds have been cleaved by ring-fission dioxygenases (Davis *et al.*, 1999; Harpel & Lipscomb, 1990*b*). Recently, a new ring-fission dioxygenase from the naphthalenesulfonate-degrading strain *Pseudaminobacter salicylatoxidans*, which oxidizes salicylate to 2-oxohepta-3,5-dienedioic acid by a novel ring-fission mechanism, has been described (Fig. 1; Hintner *et al.*, 2001, 2004).

The salicylate dioxygenase activity of *P. salicylatoxidans* BN12 is unique among the currently known ring-fission dioxygenases in that the enzyme is able to cleave various substituted salicylates that carry only a single hydroxy group and that are not activated for a ring-fission reaction by additional electron-donating substituents. Previous biochemical characterization of the salicylate dioxygenase activity from *P. salicylatoxidans* BN12 demonstrated that in addition to salicylate, the enzyme also converts gentisate, 5-aminosalicylate and



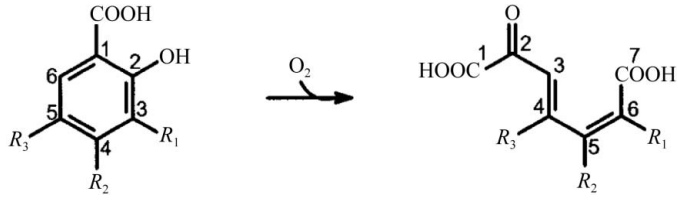


Figure 1
Salicylate dioxygenase-catalyzed oxidative cleavage of (substituted) salicylate(s): R_1, R_2, R_3 = H, NH_2, OH, CH_3, F, Cl, Br, I.

1-hydroxy-2-naphthoate, 3-amino- and 3- and 4-hydroxysalicylate, 5-fluorosalicylate, 3-, 4- and 5-chlorosalicylate, 3-, 4- and 5-bromosalicylate, 3-, 4- and 5-methylsalicylate and 3,5-dichlorosalicylate (see Fig. 1).

Sequence alignments and gel-filtration experiments suggested salicylate dioxygenase to be structurally similar to gentisate 1,2-dioxygenase from different microorganisms, such as *Comamonas testosteroni*, *C. acidovorans*, *Haloferax* sp., *Klebsiella pneumoniae*, *Moraxella osloensis* OA3, *Pseudomonas alcaligenes* and *Sphingomonas* sp. strain RW5 (Crawford *et al.*, 1975; Feng *et al.*, 1999; Fu & Oriel, 1998; Harpel & Lipscomb, 1990*a*; Hintner *et al.*, 2001, 2004; Luo *et al.*, 2006; Suarez *et al.*, 1996; Werwath *et al.*, 1998; Zhou *et al.*, 2001).

This was indicated by the size of the subunits (about 40 kDa), the structure of the holoenzyme (tetramer) and the dependence of the enzyme on Fe^{2+} ions (one iron per monomer). Nevertheless, it became evident that the ring-fission dioxygenase from *P. salicylatoxidans* was clearly different from the presently known gentisate 1,2-dioxygenases or 1-hydroxy-2-naphthoate dioxygenases because of its unique ability to oxidatively cleave salicylate and also its ability to cleave gentisate and 1-hydroxy-2-naphthoate with high catalytic efficiencies (Hintner *et al.*, 2001, 2004).

The enzyme from *P. salicylatoxidans* BN12 was heterologously expressed in *Escherichia coli* and purified as a His-tagged enzyme variant. The deduced amino-acid sequence encoded a protein with a molecular weight of 41 176 Da, which showed 28 and 31% sequence identity, respectively, to a gentisate 1,2-dioxygenase from *Pseudomonas alcaligenes* NCIMB 9867 (GenBank accession No. AAD49427) and a 1-hydroxy-2-naphthoate 1,2-dioxygenase from *Nocardioides* sp. KP7 (GenBank accession No. BAA31235) (Hintner *et al.*, 2004).

In order to allow a more detailed analysis of the relationship between the mechanistic capabilities of this particular ring-fission dioxygenase and its structural features, this enzyme was crystallized and X-ray diffraction data were collected.

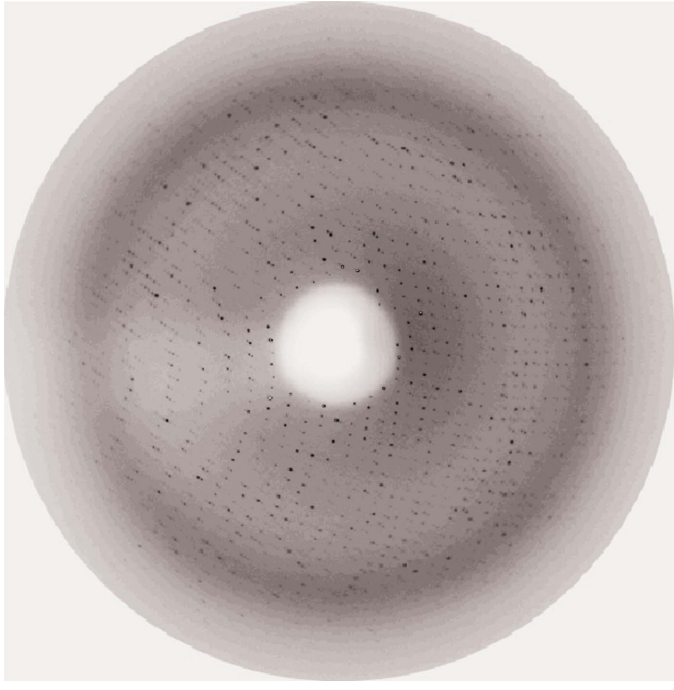


Figure 2
Area-detector frame showing the diffraction spots for crystals of salicylate 1,2-dioxygenase from *P. salicylatoxidans*.

2. Experimental procedures

2.1. Protein purification

Salicylate 1,2-dioxygenase from *P. salicylatoxidans* strain BN12 DSM 6986 was heterologously expressed in *E. coli* JM109 (pJPH100exN) as reported by Hintner *et al.* (2004). The His-tagged enzyme variant was purified, tested for activity and analyzed for purity as previously described (Hintner *et al.*, 2004).

2.2. Crystallization

The enzyme was concentrated to 19 mg ml^{-1} in 20 m*M* Tris–HCl pH 8, 100 m*M* sodium chloride using a Centricon ultraconcentrator (10 kDa molecular-weight cutoff, Amicon).

Crystallization experiments were performed using the sitting-drop vapour-diffusion method and 96-well plates (CrystalQuick, Greiner Bio-One, Germany).

Initial crystallization trials were performed using Structure Screens I and II from Molecular Dimensions Ltd and JBScreen Classic from Jena Bioscience at 277 K. Condition C2 of the JBScreen Classic 8 [12%(*w*/*v*) ethanol, 4%(*w*/*v*) PEG 400, 100 m*M* sodium acetate pH 4.6] was chosen as the most promising and was optimized by modifying the concentration of the different components and the pH of the buffer.

Diffraction-quality crystals were obtained at 277 K from a solution containing 8–12%(*w*/*v*) ethanol, 6%(*w*/*v*) PEG 400, 0.1 *M* sodium acetate pH 4.6. Drops were prepared using 1 µl protein solution mixed with 1 µl reservoir solution and were equilibrated against 100 µl precipitant solution.

2.3. X-ray data collection

A complete data set extending to a maximum resolution of 2.9 Å was collected at 100 K on EMBL beamline BW7B, Hamburg, Germany. After adding 30% glycerol to the mother liquor as a cryoprotectant, data were collected using a MAR 345 image-plate detector and a wavelength of 0.8423 Å (Fig. 2). The crystals showed no significant decay upon exposure.

3. Results

Under the optimal conditions (see §2), crystals of salicylate 1,2-dioxygenase from *P. salicylatoxidans* grow within one week at 277 K using the sitting-drop vapour-diffusion method to approximate dimensions of 0.2 × 0.2 × 0.2 mm (Fig. 3).

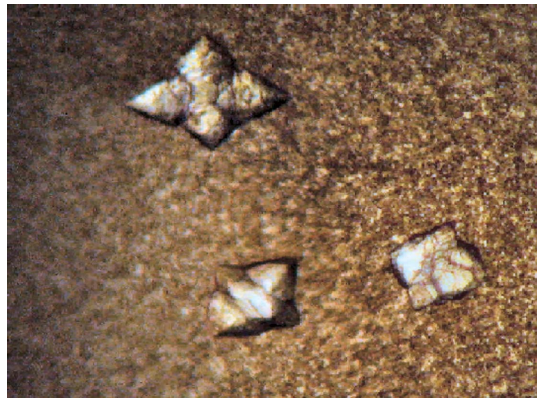


Figure 3
Microphotography of salicylate 1,2-dioxygenase from *P. salicylatoxidans* crystals obtained by the sitting-drop vapour-diffusion method.

Table 1
Crystal parameters and data-collection statistics.

Beamline	BW7B, DESY, Hamburg
Space group	$P4_32_12$ or $P4_12_12$
Unit-cell parameters	
a (Å)	133.02
c (Å)	190.75
Asymmetric unit content	1 molecule
V_M (Å^3 Da^{-1})	2.3
Solvent content (%)	47
Wavelength (Å)	0.8423
Resolution limits (Å)	20–2.9 (3.11–2.9)
Total reflections measured	749226
Unique reflections	38218
R_{sym}†	0.12 (0.47)
Multiplicity	4.8 (4.9)
Completeness (%)	99.0 (99.6)
$I/\sigma(I)$	4.4 (1.7)

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

Crystals belong to the primitive tetragonal space group $P4_32_12$ or $P4_12_12$, with unit-cell parameters a = 133.3, c = 191.51 Å. Assuming one tetramer per asymmetric unit, the solvent content is 47% of the unit cell (Matthews coefficient V_M = 2.3 Å^3 Da^{-1}; Matthews, 1968).

Data processing with *MOSFLM* and *SCALA* gave 38 218 unique reflections, an R_{sym} of 12.1% and an overall completeness of 99.0%. Statistics of the data collection and processing are reported in Table 1.

Salicylate 1,2-dioxygenase from *P. salicylatoxidans* shows sequence homology to several gentisate 1,2-dioxygenases, the structure of which is still unknown. We have attempted to carry out molecular replacement with the program *MOLREP* (Vagin & Teplyakov, 1997) using models of known extradiol dioxygenase structures, among them the quercetin 2,3-dioxygenase from *Aspergillus japonicus*, which shares the highest sequence homology with salicylate 1,2-dioxygenase from *P. salicylatoxidans* (PDB code 1gqg; 15.1% sequence identity; Steiner *et al.*, 2002). Furthermore, a homology search against protein sequences from the Protein Data Bank was carried out using *FFAS* (http://ffas.ljcrf.edu) and the structures with the highest sequence identity were used as initial models for molecular-replacement calculations. All these attempts were unsuccessful in finding a solution for salicylate 1,2-dioxygenase from *P. salicylatoxidans*.

Since salicylate 1,2-dioxygenase from *P. salicylatoxidans* contains Fe^{II} ions, it may have sufficient anomalous signal for the multiple anomalous dispersion (MAD) method. Attempts will be made to solve the structure of the enzyme by a MAD experiment using the anomalous signal of the catalytic Fe^{II}.

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