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

Salicylate 1,2-dioxygenase, a new ring-fission dioxygenase from the naphthalene-sulfonate-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, 1990b). 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 , $R_3 = \text{H}$, 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 & Oriol, 1998; Harpel & Lipscomb, 1990a; 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.

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 mM Tris-HCl pH 8, 100 mM 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 mM 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 $0.2 \times 0.2 \times 0.2 \text{ mm}$ (Fig. 3).

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

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
$R_{\text{sym}}^\dagger$	0.12 (0.47)
Multiplicity	4.8 (4.9)
Completeness (%)	99.0 (99.6)
$I/\sigma(I)$	4.4 (1.7)

$^\dagger R_{\text{sym}} = \sum |I_i - \langle I \rangle| / \sum \langle I \rangle$, 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 $V_M = 2.3$ Å³ 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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References

- Bugg, T. D. H. (2003). *Tetrahedron*, **59**, 7075–7101.
- Costas, M., Mehn, M. P., Jensen, M. P. & Que, L. (2004). *Chem. Rev.* **104**, 939–986.
- Crawford, R. L., Hutton, S. W. & Chapman, P. J. (1975). *J. Bacteriol.* **121**, 794–799.
- Davis, J. K., He, Z., Somerville, C. C. & Spain, J. C. (1999). *Arch. Microbiol.* **172**, 330–339.
- Feng, Y., Khoo, H. E. & Poh, C. L. (1999). *Appl. Environ. Microbiol.* **65**, 946–950.
- Fu, W. & Oriel, P. (1998). *Extremophiles*, **2**, 439–446.
- Harpel, M. R. & Lipscomb, J. D. (1990a). *J. Biol. Chem.* **265**, 6301–6311.
- Harpel, M. R. & Lipscomb, J. D. (1990b). *J. Biol. Chem.* **265**, 22187–22196.
- Hintner, J. P., Lechner, C., Riegert, U., Kuhm, A. E., Storm, T., Reemtsma, T. & Stolz, A. (2001). *J. Bacteriol.* **183**, 6936–6942.
- Hintner, J. P., Reemtsma, T. & Stolz, A. (2004). *J. Biol. Chem.* **279**, 37250–37260.
- Luo, S., Liu, D. Q., Liu, H. & Zhou, N. Y. (2006). *Microbiol. Res.* **161**, 138–144.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- Steiner, R. A., Kooter, I. M. & Dijkstra, B. W. (2002). *Biochemistry*, **41**, 7955–7962.
- Suarez, M., Ferrer, E. & Martin, M. (1996). *FEMS Microbiol. Lett.* **143**, 89–95.
- Vagin, A. & Teplyakov, A. (1997). *J. Appl. Cryst.* **30**, 1022–1025.
- Werwath, J., Arfmann, H. A., Pieper, D. H., Timmis, K. N. & Wittich, R. M. (1998). *J. Bacteriol.* **180**, 4171–4176.
- Zhou, N. Y., Fuenmayor, S. L. & Williams, P. A. (2001). *J. Bacteriol.* **183**, 700–708.