Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Purification, crystallization and X-ray diffraction analysis of a novel ring-cleaving enzyme ($BoxC_C$) from *Burkholderia xenovorans* LB400

Jasleen Bains and Martin J. Boulanger*

Biochemistry and Microbiology, University of Victoria, PO Box 3055 STN CSC, Victoria, BC, V8W 3P6, Canada

Correspondence e-mail: mboulang@uvic.ca

Received 18 February 2008
Accepted 18 April 2008

The assimilation of aromatic compounds by microbial species requires specialized enzymes to cleave the thermodynamically stable ring. In the recently discovered benzoate-oxidation (*box*) pathway in *Burkholderia xenovorans* LB400, this is accomplished by a novel dihydrodiol lyase ($BoxC_C$). Sequence analysis suggests that $BoxC_C$ is part of the crotonase superfamily but includes an additional uncharacterized region of approximately 115 residues that is predicted to mediate ring cleavage. Processing of X-ray diffraction data to 1.5 Å resolution revealed that $BoxC_C$ crystallized with two molecules in the asymmetric unit of the $P2_12_12_1$ space group, with a solvent content of 47% and a Matthews coefficient of 2.32 $Å^3$ Da^{-1}. Selenomethionine $BoxC_C$ has been purified and crystals are currently being refined for anomalous dispersion studies.

1. Introduction

Benzoate-containing compounds, many of which are derived from decaying plant material, are widely distributed in nature and play a key role in the global carbon cycle. The inherent thermodynamic stability of the aromatic ring restricts nonbiological turnover and consequently the removal of these compounds from the environment relies on microbial metabolic pathways. However, xenobiotic aromatic compounds in the form of industrial effluents, pesticides and solvents are relatively new to the environment and present unique challenges for microbial degradation. A promising strategy to facilitate the degradation of these pollutants is to engineer increasingly efficient metabolic pathways with broadened substrate specificity. As a prerequisite step towards the rational engineering of these biological systems, detailed biochemical and structural descriptions of the enzymes involved are essential.

Microbial degradation of aromatic compounds occurs both aerobically (Bugg, 2003; Harwood & Parales, 1996; Stanier & Ornston, 1973) and anaerobically (Evans, 1977; Evans & Fuchs, 1988; Harwood & Gibson, 1986). However, recent studies have identified a metabolic pathway in *Burkholderia xenovorans* LB400 (hereafter referred to as LB400) and *Azoarcus evansii*, termed the benzoate-oxidation (*box*) pathway, that incorporates features of both (Denef *et al.*, 2005, 2006; Mohamed *et al.*, 2001). A unique feature of LB400 is that it contains two paralogous copies of the *box* pathway: one on the chromosome (box_C) and the second on a plasmid (box_M). In each case, however, the *box* pathway catalyzes the aerobic degradation of benzoate to yield succinyl and acetyl coenzyme A (CoA), which are used as metabolic fuels in the Krebs cycle. A salient feature of this pathway is that each intermediate is processed as a CoA thioester, which is predicted to facilitate ring cleavage *via* a novel dihydrodiol lyase (2,3-dihydro-2,3-dihydroxybenzoyl-CoA lyase/hydratase; BoxC; Gescher *et al.*, 2005). Sequence analysis suggests that BoxC is a member of the crotonase superfamily. The closest homologue for which a structure has been solved is the enoyl-CoA hydratase (subunit II) from *Geobacillus kaustophilus* (PDB code 2ej5), which shows approximately 27% sequence identity over 220 residues. However, BoxC is nearly double the size of a prototypical hydratase

(Holden *et al.*, 2001) and also includes a central region of approximately 115 residues that has no significant sequence similarity to other members of the crotonase superfamily. Intriguingly, it is this region that is predicted to catalyze the cleavage of the uncommon alicyclic dihydroxylated benzoate ring using $\Delta^{3,5},\Delta^{2,4}$-dienoyl-coenzyme A isomerase-like and 2-ketocyclohexylcarboxyl-CoA hydrolase-like activities (Gescher *et al.*, 2005). No structural studies have been reported for a BoxC enzyme from any organism and as a result details of the features that govern catalysis are undefined.

A key step in the assimilation of benzoates by the recently discovered benzoate-oxidation (*box*) pathway is the cleavage of the dihydroxylated benzoate ring by BoxC. As a first step towards establishing the structural and mechanistic properties of these novel ring-cleaving enzymes, expression, purification and preliminary high-resolution (1.5 Å) X-ray data analysis was carried out on the chromosomally encoded BoxC ($BoxC_C$) from LB400. The preliminary characterization reported here represents a significant step forward in establishing a structural paradigm for aerobic benzoate degradation by the *box* pathway.

2. Materials and methods

2.1. Cloning, expression and purification

The *boxc*$_c$ (chromosomally encoded *boxc*) gene was amplified from LB400 genomic DNA and cloned into pET-28a (Novagen) in frame with an N-terminal hexahistidine tag. Sequence analysis confirmed that no mutations were introduced during amplification. Expression of recombinant $BoxC_C$ was performed in *Escherichia coli* BL21 Star (DE3) cells (Invitrogen) grown in 2×YT media (EMD chemicals) supplemented with 50 µg ml^{-1} kanamycin (Sigma). Transformed cells were initially grown at 310 K to an OD_{600} of 1, at which point the temperature was lowered to 300 K and $BoxC_C$ expression was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.75 m*M*. After 12 h, the cells were harvested by centrifugation at 9000*g* for 15 min and the pellet was resuspended in 20 m*M* HEPES pH 7.5, 15 m*M* imidazole, 3 m*M* β-mercaptoethanol and 500 m*M* NaCl. The cells were lysed using a French press (SLM Instruments) and the insoluble fraction was removed by centrifugation at 20 000*g* for 45 min. The supernatant was applied onto Ni–NTA resin (Qiagen) and washed extensively with buffer *A* (20 m*M* HEPES pH 7.5, 500 m*M* NaCl and 3 m*M* β-mercaptoethanol) supplemented with 20 m*M* imidazole and $BoxC_C$ was eluted with buffer *A* supplemented with 250 m*M* imidazole. SDS–PAGE was used to check the purity of $BoxC_C$ prior to proteolytic removal of the hexahistidine tag by thrombin. In its mature form following thrombin cleavage, $BoxC_C$ includes an additional three amino acids at the N-terminus with Gly and Ser derived from the thrombin site and His contributed from the *Nde*I site. No additional residues were added at the C-terminus. In the final step, $BoxC_C$ was purified on a Superdex S-200 16/60 HiLoad size-exclusion column (GE Healthcare) equilibrated with buffer *B* (20 m*M* HEPES pH 7.5, 150 m*M* NaCl and 3 m*M* β-mercaptoethanol). Fractions were analyzed by SDS–PAGE, pooled based on purity and concentrated to 13 mg ml^{-1} in buffer *B* as determined by A_{280}.

Expression and purification of selenomethionine $BoxC_C$ was carried out as described for native $BoxC_C$ with the following changes: the expression vector was transformed into *E. coli* 834 (DE3) (a

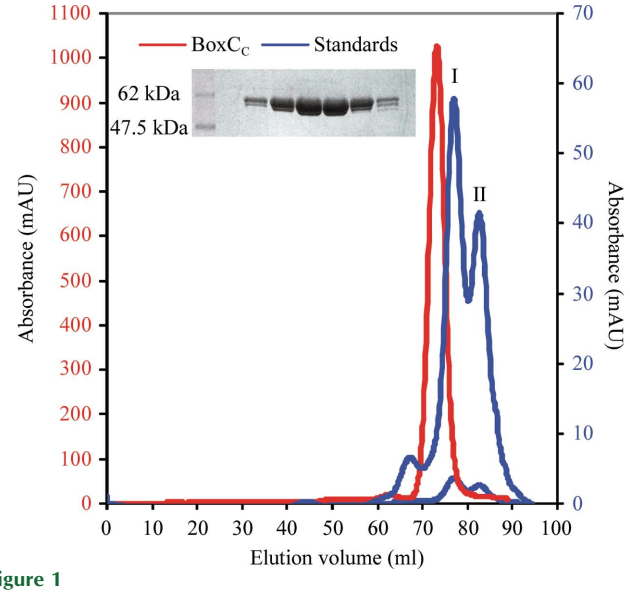
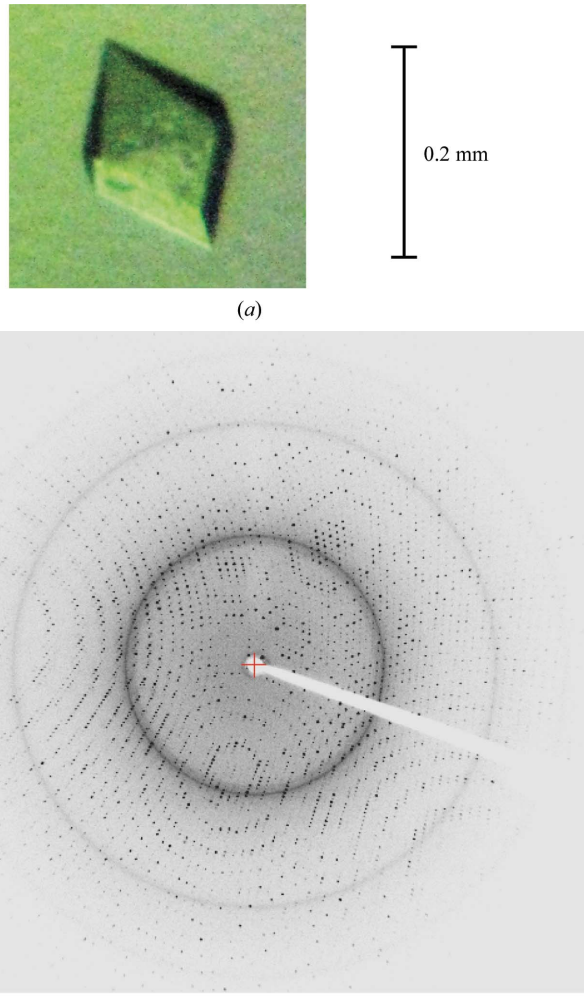


Figure 1
Superdex 200 16/60 gel-filtration analysis showing that $BoxC_C$ (red peak) forms a stable dimer of ∼120 kDa in solution. The blue peaks represent protein standards: peak I, conalbumin (75 kDa); peak II, ovalbumin (43 kDa). Inset, SDS–PAGE analysis of the column fractions, with the $BoxC_C$ monomer migrating at approximately 60 kDa (the expected MW of the monomer is 61 579 Da).



(*a*)

(*b*)

Figure 2
(*a*) Native $BoxC_C$ crystal grown in 23% PEG 3350 buffered with 100 m*M* Tris pH 8.5. (*b*) X-ray diffraction image of the native $BoxC_C$ crystal collected to 1.5 Å resolution.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell (1.55–1.5 Å).

Space group	$P2_12_12_1$
Unit-cell parameters (Å, °)	a = 85.16, b = 99.85, c = 136.73, $\alpha = \beta = \gamma = 90$
Wavelength (Å)	1.54
Resolution (Å)	34.18–1.5
Measured reflections	1181949
Unique reflections	183917
Redundancy	6.43 (4.13)
Completeness	98.8 (88.7)
$I/\sigma(I)$	13.4 (3.2)
$R_{\rm merge}$† (%)	0.062 (0.358)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the average over all observations of reflection hkl.

methionine auxotroph; Novagen) and grown in SelenoMet medium (AthenaES) supplemented with L-selenomethionine to a final concentration of 40 µg ml^{-1} (AthenaES).

2.2. Crystallization

Initial crystallization trials were set up with commercial screens (Wizard I, II and III from Emerald Biosystems and Index and PEG/Ion Screens from Hampton Research) in 96-well plates (Axygen Biosciences). The final drops consisted of 1.5 µl protein solution (at 13 mg ml^{-1}) and 1.5 µl reservoir solution and were equilibrated against 100 µl reservoir solution. Small crystals of native BoxC$_C$ were observed in 23% PEG 3350 with 100 m*M* Tris pH 8.5 after 3 d at 293 K. Two rounds of streak-seeding were required to sufficiently increase the crystal size (0.2 × 0.1 × 0.2 mm) and quality to enable data collection. For the streak-seeding experiment, approximately a dozen small crystals (0.05 × 0.05 × 0.1 mm) were harvested into 50 µl reservoir solution and crushed with a seed bead (Hampton Research, USA). 0.3 µl of the seed mix was then added to each sitting drop pre-equilibrated with protein and reservoir solution (1:1) with a 3 µl total volume. Optimization of selenomethionine BoxC$_C$ crystals is currently under way.

2.3. Data collection and processing

A single native BoxC$_C$ crystal (Fig. 2*a*) was looped into cryoprotectant consisting of mother liquor supplemented with 20% glycerol for 30 s and flash-cooled directly in the cryostream (100 K). Diffraction data were collected on a Rigaku R-AXIS IV^{++} area detector coupled to an MM-002 X-ray generator with Osmic 'blue' optics and an Oxford Cryostream 700. Each of the 450 images was exposed for 180 s over an oscillation range of 0.5° (Fig. 2*b*). Diffraction data to 1.5 Å were processed using *CrystalClear*/*d***TREK* (Pflugrath, 1999). Data-collection statistics are presented in Table 1.

3. Results and discussion

The novel ring-cleaving enzyme BoxC$_C$ from LB400 was successfully cloned, recombinantly expressed, purified and crystallized. Relative to protein standards, the elution profile of BoxC$_C$ from a Superdex 200 size-exclusion column showed the enzyme to be a stable dimer in solution with a molecular weight of approximately 120 kDa (Fig. 1), which is consistent with its homologue from *A. evansii* (Gescher *et al.*, 2005). The gel-filtration fractions were analyzed by SDS–PAGE, which showed a predominant band at approximately 60 kDa as expected for the BoxC$_C$ monomer (61 579 Da). Streak-seeding produced high-quality crystals that diffracted to 1.5 Å resolution. Processing of the data (Table 1) showed that BoxC$_C$ crystallized as a dimer in the asymmetric unit of the $P2_12_12_1$ unit cell, which was consistent with the observation of a non-origin peak in the self-rotation function, with a solvent content of 47% and a Matthews coefficient of 2.32 Å^3 Da^{-1} (Matthews, 1968). Scaling and merging of the data resulted in an overall $R_{\rm merge}$ of 6.2%, with a value of 35.8% in the highest resolution shell (1.55–1.5 Å). Ultimately, structure solution of BoxC$_C$ will require experimental phases. To this end, selenomethionine BoxC$_C$ protein was purified and crystals are currently being refined for anomalous dispersion synchrotron data collection.

This work was supported by a research grant from the Natural Sciences and Engineering Research Council of Canada to MJB. JB was supported by a University of Victoria Graduate fellowship and MJB is a Canadian Institutes of Health Research New Investigator and a Michael Smith Foundation for Health Research Scholar.

References

Bugg, T. D. H. (2003). *Tetrahedron*, **59**, 7075–7101.
Denef, V. J., Klappenbach, J. A., Patrauchan, M. A., Florizone, C., Rodrigues, J. L., Tsoi, T. V., Verstraete, W., Eltis, L. D. & Tiedje, J. M. (2006). *Appl. Environ. Microbiol.* **72**, 585–595.
Denef, V. J., Patrauchan, M. A., Florizone, C., Park, J., Tsoi, T. V., Verstraete, W., Tiedje, J. M. & Eltis, L. D. (2005). *J. Bacteriol.* **187**, 7996–8005.
Evans, W. C. (1977). *Nature (London)*, **270**, 17–22.
Evans, W. C. & Fuchs, G. (1988). *Annu. Rev. Microbiol.* **42**, 289–317.
Gescher, J., Eisenreich, W., Worth, J., Bacher, A. & Fuchs, G. (2005). *Mol. Microbiol.* **56**, 1586–1600.
Harwood, C. S. & Gibson, J. (1986). *J. Bacteriol.* **165**, 504–509.
Harwood, C. S. & Parales, R. E. (1996). *Annu. Rev. Microbiol.* **50**, 553–590.
Holden, H. M., Benning, M. M., Haller, T. & Gerlt, J. A. (2001). *Acc. Chem. Res.* **34**, 145–157.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Mohamed, M. E., Zaar, A., Ebenau-Jehle, C. & Fuchs, G. (2001). *J. Bacteriol.* **183**, 1899–1908.
Pflugrath, J. W. (1999). *Acta Cryst.* D**55**, 1718–1725.
Stanier, R. Y. & Ornston, L. N. (1973). *Adv. Microb. Physiol.* **9**, 89–151.