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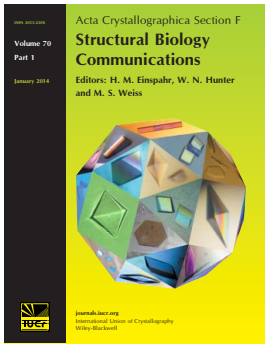

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Crystallization and preliminary X-ray characterization of the 2,4′-dihydroxyacetophenone dioxygenase from *Alcaligenes* sp. 4HAP

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The enzyme 2,4′-dihydroxyacetophenone dioxygenase (or DAD) catalyses the conversion of 2,4′-dihydroxyacetophenone to 4-hydroxybenzoic acid and formic acid with the incorporation of molecular oxygen. Whilst the vast majority of dioxygenases cleave within the aromatic ring of the substrate, DAD is very unusual in that it is involved in C—C bond cleavage in a substituent of the aromatic ring. There is evidence that the enzyme is a homotetramer of 20.3 kDa subunits each containing nonhaem iron and its sequence suggests that it belongs to the cupin family of dioxygenases. By the use of limited chymotrypsinolysis, the DAD enzyme from *Alcaligenes* sp. 4HAP has been crystallized in a form that diffracts synchrotron radiation to a resolution of 2.2 Å.

1. Introduction

The enzyme 2,4′-dihydroxyacetophenone dioxygenase (or DAD) catalyses the conversion of 2,4′-dihydroxyacetophenone, a breakdown product of lignin, into 4-hydroxybenzoic acid and formic acid with the incorporation of molecular oxygen (Fig. 1). As a bacterial dioxygenase, DAD plays an important environmental role in the aerobic catabolism of aromatic compounds. Expression of enzymes like this one in appropriately engineered microorganisms has potential for the removal of aromatic pollutants from soil and groundwater, and in the production of chemicals, for example in biotransformations. Indeed, the bacterial species from which the enzyme originates (*Alcaligenes*) has been used industrially for biotransformations in the production of unusual amino acids and polyhydroxybutanol.

Whilst the vast majority of dioxygenases cleave within the aromatic ring, DAD is very unusual in that it is involved in C—C bond cleavage in a substituent of the aromatic ring. The DAD enzyme from *Alcaligenes* sp. 4HAP has been cloned and expressed in *Escherichia coli* (Hopper & Kaderbhai, 1999). Each subunit of the DAD enzyme has a molecular mass of 20.3 kDa and consists of 177 amino acids and, like many other dioxygenases, it contains iron. The iron is of unknown redox state (although the enzyme is colourless) and is not lost during prolonged dialysis, suggesting that it is tightly bound. There is evidence that the enzyme is a homotetramer of subunits containing nonhaem iron and the sequence suggests that the enzyme belongs to the cupin family of dioxygenases. The biochemical properties of the *Burkholderia* sp. AZ11 enzyme have been studied in detail recently and sequence comparisons have suggested that a number of conserved histidine and carboxylate residues may be important for iron binding (Enya *et al.*, 2012).

The DAD enzyme is involved in the bacterial breakdown of 4-hydroxyacetophenone (Hopper *et al.*, 1985). This compound in turn is an intermediate in the degradation of 4-ethylphenol (Darby *et al.*, 1987) and is related to other intermediates occurring in the breakdown of the multitude of naturally occurring aromatic compounds, found mainly as plant products, but also as manmade compounds either deliberately or accidentally released by the chemical industry. The most abundant of the plant products is lignin, which constitutes between 18 and 35% of the dry weight of plant material. The ability to breakdown lignin, which has a polyaromatic structure, and other

aromatic plant products is a unique feature of microorganisms, offering enormous potential for exploitation as a source of organic compounds.

Oxygenases like DAD are, by definition, enzymes that catalyse reactions in which O atoms are incorporated into the aromatic substrates to facilitate their metabolism. This was demonstrated by isotope experiments using $^{18}O_2$ in which the products of the reaction were shown to contain both equivalents of labelled oxygen (Hayaishi, 1962, 1966). These enzymes fall into two classes, namely the monooxygenases and dioxygenases, which differ in the number of oxygen equivalents incorporated. Generally, the metabolism of aromatic compounds involves ring opening, which usually proceeds by insertion of at least two hydroxyl groups into the ring either *ortho* or *para* to each other. The ring is then cleaved either at the C—C bond between the two hydroxyls (catalysed by an intradiol dioxygenase) or at an adjacent C—C bond (catalysed by an extradiol dioxygenase). To break the bonds in aliphatic substituents of the ring, or in aliphatic compounds in general, lyase and hydrolase reactions are commonly used in nature, although sometimes monooxygenases are involved. Thus, whilst the vast majority of dioxygenases cleave within the aromatic nucleus, the enzyme of this study (DAD) is one of the few that are involved in C—C bond cleavage in a substituent of the aromatic ring (see Fig. 1). As with the ring-opening enzymes, isotope experiments using $^{18}O_2$ have confirmed that the DAD enzyme is a true dioxygenase, *i.e.* both atoms of O_2 are incorporated into the substrate stoichiometrically (Hopper, 1986).

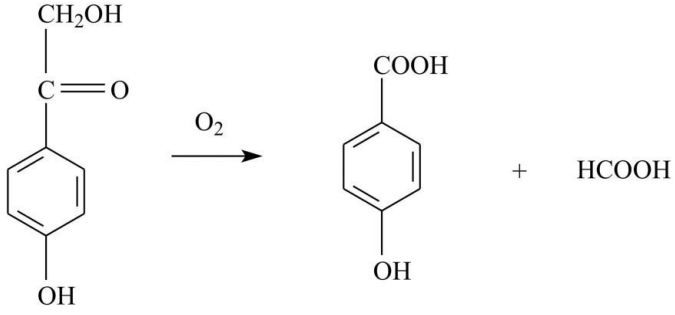

Figure 1
The reaction catalysed by 2,4′-dihydroxyacetophenone dioxygenase (DAD). The enzyme has a high affinity for oxygen, which is used for C—C bond cleavage of the substrate (2,4′-dihydroxyacetophenone) yielding 4-hydroxybenzoic acid and formate.

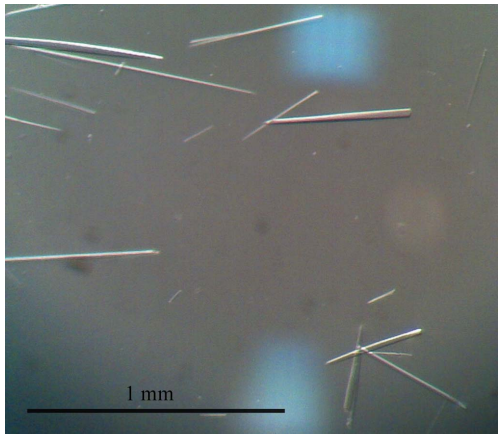

Figure 2
Crystals of DAD that were obtained following limited chymotrypsin digestion. These needle-like crystals are up to 1 mm in length but are around 10 µm in thickness.

Our aim is to eventually solve the crystal structure of the DAD enzyme and to analyse complexes of the enzyme with inhibitors and the reaction products to characterize its mechanism of action. To this end, we have expressed and crystallized the enzyme in a form that diffracts synchrotron radiation to 2.2 Å resolution.

2. Expression, purification and crystallization

The DAD gene in the original plasmid described by Hopper & Kaderbhai (1999) was amplified by standard PCR methods with primers designed to introduce *Nde*I and *Bam*HI restriction sites at the 5′ and 3′ ends of the gene, respectively. The corresponding forward and reverse primer sequences were as follows: 5′-TAGCCTGGATCTAGA**CATATG**GTCGCTAACGCAATCAGCGAG and 5′-CAAGCTT**GGATCC**TCACTAGCGGAAAAGAGTAACGACAAGGTC. The *Taq*-amplified DNA was digested with the above enzymes and ligated using standard cloning methods into the expression vector pET-15b (Novagen), which conferred the protein with a thrombin-cleavable N-terminal hexahistidine tag. The sequence of the resulting construct was confirmed by DNA sequencing (Sequencing Service, University of Dundee, Scotland; http://www.dnaseq.co.uk).

The protein was expressed in *E. coli* BL21(DE3) cells transformed with the above plasmid and grown in 0.5 l cultures of LB medium in the presence of 50 µg ml^{-1} ampicillin in shaking flasks with orbital agitation at 120 rev min^{-1} to mid-log phase prior to induction with 1 m*M* isopropyl *β*-D-1-thiogalactopyranoside (IPTG) and overnight growth at 37°C. The cells were harvested by centrifugation and stored frozen. Following freeze–thawing and sonication of the cell pellet, ultracentrifugation of the lysate followed by SDS–PAGE analysis showed that the His-tagged DAD protein was overexpressed in soluble form. The protein was purified from the supernatant by nickel-affinity chromatography using HisTrap 1 ml columns (GE Healthcare). Since the purified protein aggregated upon storage in 0.5 *M* imidazole following elution from the nickel column, it was necessary to dilute it immediately in sonication buffer (50 m*M* Tris pH 7.5, 100 m*M* NaCl, 1 m*M* *β*-mercaptoethanol) prior to further buffer exchange using the same buffer in a centrifugal concentrator. The yield of affinity-purified protein was estimated to be 20 mg per litre of *E. coli* cell culture.

To obtain crystals of sufficient size for X-ray analysis, it was necessary to mix the purified enzyme at a concentration of 5 mg ml^{-1} with a small amount of chymotrypsin (added to give a DAD:chymotrypsin mass ratio of 50:1) and incubate the mixture at room temperature for 3 h prior to setting up hanging-drop crystallization trials following the procedures of Dong *et al.* (2007) and Wernimont & Edwards (2007). Further details are given in the Supporting Information[1]. No efforts were made to specifically remove the His tag with thrombin, although this approach has been tried previously as a possible means of improving the crystallization, albeit without success.

Other prior efforts to crystallize the protein had involved the use of a range of other expression constructs, including the Hopper & Kaderbhai (1999) construct along with an untagged form made with pET-11a (Novagen) and a GST-tagged form made using pRL-296a (a kind gift from M. Cygler, University of Montreal). These efforts usually yielded extremely small, thin and hollow needles that often diffracted to only 10 or 20 Å resolution at the synchrotron. However, a number of more promising crystal forms were obtained from these

[1] Supporting information has been deposited in the IUCr electronic archive (Reference: FW5455).

studies, as described in Beaven (2005) and Bowyer (2009). Although some of these crystal forms yielded promising diffraction data, they did not allow the structure to be solved.

Interestingly, chymotrypsinolysed DAD protein produced from the pET-15b construct yielded larger and cleaner-looking crystals under a range of conditions in Molecular Dimensions Structure Screens (SS) 1 and 2 (see Fig. 2). The optimum conditions in terms of diffraction resolution were found to be 10%(*w*/*v*) PEG 1K and 10% PEG 8K (SS2 condition 46), although several other conditions (14, 21, 26 and 33 from SS1 as well as 4 from SS2) gave crystals of similar visual quality.

Following on from these findings, expression of selenomethionine-substituted protein with the pET-15b construct by growth of the cells in minimal medium supplemented with selenomethionine was undertaken. However, crystallization trials with chymotrypsin-treated selenomethionyl DAD protein did not yield any diffraction-quality crystals.

3. X-ray data collection and analysis

Crystals of the native chymotrypsinolysed His-tagged DAD were transferred to the well solution, where the glycerol concentration was raised in steps to approximately 30%(*v*/*v*). The cryoprotected crystals were then mounted in loops before being flash-cooled with an Oxford Cryosystems cryostream. Being very needle-like, the crystals had a tendency to bend and break or twist during any of these manipulations. Whilst the majority of crystals were around 300–600 µm in length, they were only around 10–15 µm thick and generally diffracted synchrotron radiation to at best 2.5–3.0 Å resolution, with a high rate of radiation damage. However, in one favourable case (Fig. 3) 90° of data were collected to a resolution of 2.2 Å on the BM14U beamline at the ESRF, Grenoble, France using a MAR CCD detector. This data set was obtained using a wavelength of 1.072 Å, an oscillation angle of 1°, an exposure time of 20 s per image and a crystal-to-detector distance of 252.6 mm (Fig. 4). The data were processed with *iMosflm* (Battye *et al.*, 2011; Powell *et al.*, 2013) and *SCALA* (Evans, 2006) in the *CCP*4 suite (Winn *et al.*, 2011), which suggested that the space group was either $P6_122$ or $P6_522$, with unit-cell parameters $a = b = 82.6$, $c = 114.0$ Å. Scaling the data in point group 622 gave an R_{merge} of 13.6% and an R_{meas} of 14.3% with a 9.8-fold multiplicity; other processing statistics are shown in Table 1. Whilst the R_{merge} is slightly high, the data have excellent consistency by the criterion of Karplus & Diederichs (2012). Using the method of Matthews (1968), as implemented by Kantardjieff & Rupp (2003), it was estimated that the crystals have a single DAD monomer per crystallographic asymmetric unit and a solvent content of 56%. Analysis of the structure by molecular replacement is in progress and preliminary results indicate that the correct space group is $P6_522$. It is hoped that analysis of the structure will give key insights into the mechanism of this very unusual cupin-family dioxygenase.

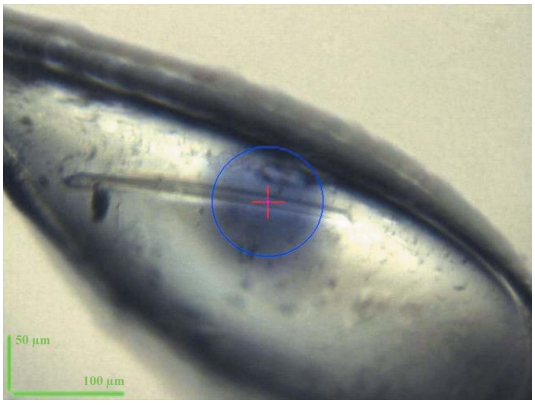

Figure 3
The DAD crystal which yielded synchrotron X-ray data to 2.2 Å resolution at the ESRF, Grenoble. The crystal was cryoprotected in 30% glycerol and maintained at −173°C for data collection.

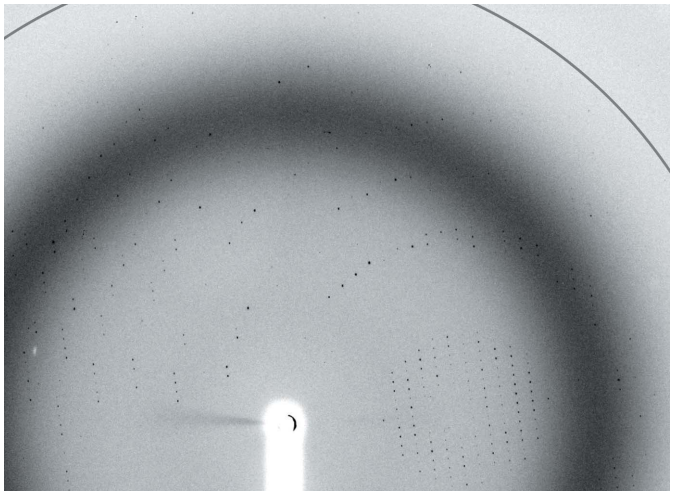

Figure 4
A diffraction image of DAD which was obtained on BM14U at the ESRF. The grey circle indicates a resolution of 2.3 Å and data-processing statistics are shown in Table 1.

Table 1
Data-collection and processing statistics for 2,4′-dihydroxyacetophenone dioxygenase from *Alcaligenes* sp. 4HAP.

Values in parentheses are for the outer resolution shell.

Beamline	BM14U, ESRF
Wavelength (Å)	1.072
Space group	$P6_522$
Unit-cell parameters	
$a = b$ (Å)	82.6
c (Å)	114.0
Mosaic spread (°)	0.18
Resolution (Å)	57.0–2.2 (2.3–2.2)
R_{merge}† (%)	13.6 (61.9)
R_{meas}‡ (%)	14.3 (66.8)
$CC_{1/2}$§ (%)	99.6 (80.1)
Completeness (%)	86.3 (49.4)
Average $I/\sigma(I)$	12.6 (3.1)
Multiplicity	9.8 (7.1)
No. of observed reflections	103761 (5973)
No. of unique reflections	10549 (836)
Wilson plot B factor (Å^2)	29.2
Solvent content (%)	56.2
No. of molecules per asymmetric unit	1

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$. ‡ $R_{meas} = \sum_{hkl}\{N(hkl)/[N(hkl)-1]\}^{1/2}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $\langle I(hkl)\rangle$ is the mean intensity of the $N(hkl)$ observations $I_i(hkl)$ of each unique reflection hkl after scaling. § $CC_{1/2}$ values are the half-set correlation coefficients, as described by Karplus & Diederichs (2012).

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