Crystallization and preliminary X-ray diffraction studies of the oxygenating subunit of 3,6-diketocamphane monooxygenase from *Pseudomonas putida*

EMMA J. MCGHIE, MICHAIL N. ISUPOV, EWALD SCHRÖDER AND JENNIFER A. LITTLECHILD* *at Departments of Chemistry and Biological Sciences, University of Exeter, Exeter EX4 4QD, England. E-mail: jalittle@ex.ac.uk*

Abstract

The oxygenating constituent of the 3,6-diketocamphane monooxygenase isozyme from *Pseudomonas putida* NCIMB 10007 has been crystallized under two different conditions. Crystals were initially grown from polyethylene glycol (PEG) 8000 and sodium acetate using the vapour-phase diffusion method. The crystals were of orthorhombic $P2_12_12_1$ space group, with cell dimensions $a = 55.8$, $b = 94.5$ and $c = 163.7$ Å and diffracted to 2.8 Å resolution. More recently, improved crystals, which diffracted beyond 2 Å, have been grown from ammonium sulfate. These crystals also belong to the orthorhombic $P2_12_12_1$ space group, with cell dimensions of $a = 54.6$, $b = 93.2$ and $c = 154.1$ Å. A full native data set to 2.5 Å resolution has been collected from the ammonium sulfate grown crystals.

1. Introduction

It is known that *Pseudomonas putida* NCIMB 10007 is able to grow on either enantiomer of camphor as the sole carbon source (LeGall *et al.*, 1963). Whereas the metabolism of (+)-camphor has been widely researched and reviewed (Conrad *et al.*, 1965; Gunsalus & Marshall, 1971; Taylor & Trudgill, 1986), there have been comparatively few studies of the fate of the complementary optical antipode (Williams, 1991; Jones *et al.*, 1993). It has been established that various monooxygenases play key roles in the dissimilation of both camphor enantiomers.

Both (+)- and (−)-camphor are initially hydroxylated by a shared cytochrome P450-dependent monooxygenase induced by growth on either enantiomer (Fig. 1). However, the subsequent oxidative steps, including the Baeyer–Villiger monooxygenase-dependent ring-cleavage reaction that results in the formation of monocyclic pathway intermediates from diketocamphane precursors are catalysed by enantiocomplementary enzymes (Fig. 1). These are induced irrespective of the camphor enantiomer used for growth (Taylor & Trudgill, 1986; Jones *et al.*, 1993).

Two diketocamphane monooxygenases from *P. putida* have been found to catalyse a number of oxygenative biotransformations with various natural and synthetic ketones. These reactions confirmed that each of the two isozymes possesses distinct enantioselective properties and can, in some cases, be exploited to undertake key reactions in the chemoenzymatic synthesis of commercially useful products (Gagnon *et al.*, 1994; Grogan *et al.*, 1992, Grogan, Roberts & Willetts, 1993; Grogan, Roberts, Wan *et al.*, 1993).

Previous studies have suggested that both the 2,5-diketocamphane monooxygenase (Williams, 1991; Grogan, 1995) and the 3,6-diketocamphane monooxygenase (Jones *et al.*, 1993; Grogan, 1995) are composed of a loose trimeric complex. Each

complex consists of an NADH dehydrogenase, which may be common to both flavoproteins, and a homodimer of identical oxygenating subunits, that determine the specificity of the isozymes. The oxygenating subunits have a monomeric subunit molecular weight of 40 and 42 kDa for the 2,5- and 3,6-diketocamphane monooxygenase oxygenating subunits, respectively. N-terminal sequence analysis of both types of oxygenating subunits show the enzymes to be related but clearly different proteins. Some limited sequence homology exists between the monooxygenase oxygenating subunits and luciferase from *Vibrio harveyii*.

No structural information on the diketocamphane monooxygenase enzymes has been reported to date. Structural and mechanistic comparisons of the diketocamphane monooxygenase isozymes and details of their active sites will enable the rational design of novel substrates as well as the modification of the substrate specificity of the enzymes by site-directed mutagenesis. This will expand the application of one or both isozymes for useful large-scale biotransformation reactions. Here we present the crystallization of the oxygenating component of the 3,6-diketocamphane monooxygenase.

2. Materials and methods

Pseudomonas putida NCIMB 10007 cells (24 l) were grown on (±)-camphor, harvested and broken by sonication (McGhie & Littlechild, 1996). The crude protein extract was made 50%(*w/v*) saturated with respect to enzyme-grade ammonium sulfate and the resulting supernatant was then taken to 75% saturation. The resulting pellet was dissolved in buffer *A* [21 m*M* KH_2PO_4/K_2HPO_4, pH 7.1, 6 m*M* β-mercaptoethanol, 0.1 m*M* EDTA, 10 μ*M* phenylmethylsulfonylfluoride (PMSF) and 20 μ*M* benzamidine (BAM)] and dialysed overnight against the same buffer. This was then applied directly to a pre-equilibrated Fast-flow-Q anion-exchange column (Pharmacia; 6 × 18 cm) in buffer *A*. The protein was eluted with a 1.5 l linear gradient of 0–0.45 *M* potassium chloride in buffer *A*.

The active fractions corresponding to the 3,6-diketocamphane monooxygenase eluted at 0.3–0.35 *M* KCl and were pooled and concentrated by precipitation with 80%(*w/v*) ammonium sulfate. The enzyme was then dissolved in a minimal amount of buffer *A* and dialysed overnight in 5 l of the same buffer. The dialysate was then directly loaded onto a pre-equilibrated Mono-Q anion-exchange column (Pharmacia; 0.5 × 5 cm) in buffer *A*. The enzyme was eluted using a 50 ml linear gradient of 0–0.45 *M* potassium chloride in buffer *A*. Active fractions were again pooled and concentrated by precipitation with 80%(*w/v*) ammonium sulfate as above. The resulting pellet was resuspended in buffer *B* (21 m*M* KH_2PO_4/K_2HPO_4, pH 7.1, 5% $(NH_4)_2SO_4$, 6 m*M* β-mercap-

toethanol, 0.1 mM EDTA, 1 × 10^{-5} M PMSF and 2 × 10^{-5} M BAM) and loaded onto a pre-equilibrated Superose 12 gel-filtration column (Pharmacia; 3.5 × 32 cm) with buffer B. The active fractions were again pooled.

2.1. Activity assays

The activity of 3,6-diketocamphane monooxygenase was assayed using a modification of the method of Jones *et al.* (1993). A standard 1 ml volume was used for each assay, containing 120 µmol KH$_2$PO$_4$/K$_2$HPO$_4$ buffer, pH 7.1, and 0.15 µmol NADH. Once an appropriate amount of protein solution was added, the endogenous change in the extinction of NADH was followed for 30 s against a blank (buffer and NADH) at 340 nm. A subsequent addition of 1 µmol (−)-camphor was made and the ensuing oxidation of NADH was measured for a further minute. The final activity measurement was the difference between the two recorded activities.

A gas-chromatography assay was also used to identify the activity of the 3,6-diketocamphane monooxygenase oxygenating component in later stages, as the prior purification steps
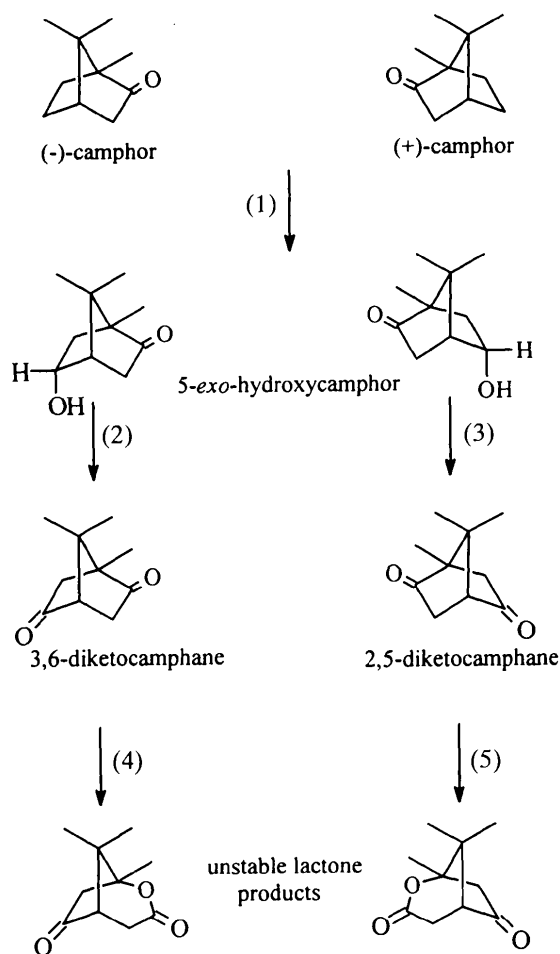

Fig. 1. The camphor degradation pathway. The following enzymes are indicated: (1), 5-exo-hydroxylase (P450$_{CAM}$); (2) and (3), exo-hydroxycamphor dehydrogenases; (4), 2,5-diketocamphane mono-oxygenase; (5), 3,6-diketocamphane monooxygenase.

resulted in the removal of the NADH dehydrogenase and so no activity of the active complex was observed using the assay described above. This method monitors the activity of the subunit to specifically lactonize the (−)-camphor substrate, which will still minimally occur in the absence of the NADH dehydrogenase. 200 µl of the relevant column fraction was mixed with 5 nmol FMN and 0.2 µmol NADH, and 0.2 µmol of (−)-camphor was added. This tube was subsequently shaken at 150 rev min^{-1} for 1 h at room temperature. Extraction was carried out by adding 200 µl of ethyl acetate, homogenizing the mixture thoroughly and subsequently centrifuging the mixture for 5 min at 13 500 rev min^{-1}. A 1 µl aliquot of the top organic layer was then removed and analysed using a Shimadzu GC-14A FID gas chromatograph equipped with a capillary column (BP1, 25 m). The column was heated to 473 K, with the detector and injector temperatures being set at 523 and 473 K, respectively.

2.2. Crystallization 1

The dimeric oxygenating component was concentrated in a Centricon concentrator (Millipore). A buffer exchange was carried out by the addition of 4 × 2.5 ml of buffer C (10 mM PIPES, pH 6.5, containing 1 mM DTT). After concentration to approximately 10 mg ml^{-1}, the protein was crystallized by vapour diffusion using the hanging-drop technique. The hanging drops (8 µl protein, 1 µl 40% PEG 8000 and 1 µl 2 M sodium acetate) were suspended in sealed compartments above a 15%(w/v) solution of PEG 8000 containing 0.2 M sodium acetate and incubated at 290 K.

2.3. Crystallization 2

The dimeric oxygenating component was concentrated in a Centricon concentrator (Millipore). A buffer exchange was carried out by the addition of 4 × 2.5 ml of buffer D (50 mM PIPES, pH 6.5, containing 1 mM DTT). The crystals were again grown by the hanging-drop vapour-diffusion method. A drop containing 9 µl of an 8 mg ml^{-1} protein solution and 1 µl saturated ammonium sulfate was equilibrated over a reservoir of 50 mM PIPES, pH 6.5, and 50% ammonium sulfate at 290 K.

2.4. X-ray data collection

All data were collected at the Daresbury Synchrotron using MAR image plates. Data for the PEG-grown crystals were collected on beamline 7.2 using a wavelength of 1.448 Å. Data for the ammonium sulfate grown crystals were collected on beamline 9.5 using a wavelength of 1.000 Å. Diffraction was recorded at room temperature for the PEG-grown crystals and under cryogenic conditions for the ammonium sulfate grown crystals. The data were processed using the *DENZO* suite of programs (Otwinowski & Minor, 1997).

3. Results and discussion

It was found that a similar crystal form was grown using the two different conditions described below. The first crystals of the 3,6-diketocamphane monooxygenase oxygenating component grew from 0.2 M sodium acetate and 15% PEG 8000 in about 1–2 weeks and were typically thin plate-shaped crystals that were 0.5 × 0.3 × 0.05 mm in dimensions (Fig. 2a). These were harvested into a mother liquor containing 20% PEG 8000 and 0.5 M sodium acetate. Large crystals were difficult to reproduce and were unstable on exposure to X-rays. This

allowed only 20° of data to be collected to 2.8 Å resolution at room temperature at the synchrotron radiation source. It was found that the crystals belong to the orthorhombic space group $P2_12_12_1$ with unit-cell dimensions $a = 55.8$, $b = 94.5$ and $c = 163.7$ Å. Assuming the subunit molecular weight to be 42 kDa, the crystal can contain two subunits per asymmetric unit with a solvent content of 52%, or one subunit with a solvent content of 76% (Matthews, 1968). Provided the 3,6-diketocamphane monooxygenase molecule is still dimeric in the crystal, two subunits per asymmetric unit seems to be plausible, as there are no proper rotational axes in this crystal space group.

The crystals of the second form had a rod-shaped morphology and were much thicker (Fig. 2b). They grew from 50 mM PIPES, pH 6.5, and 50% ammonium sulfate over several months. These were harvested into a mother liquor containing 30% glycerol, 50 mM PIPES, pH 6.5, and 55%
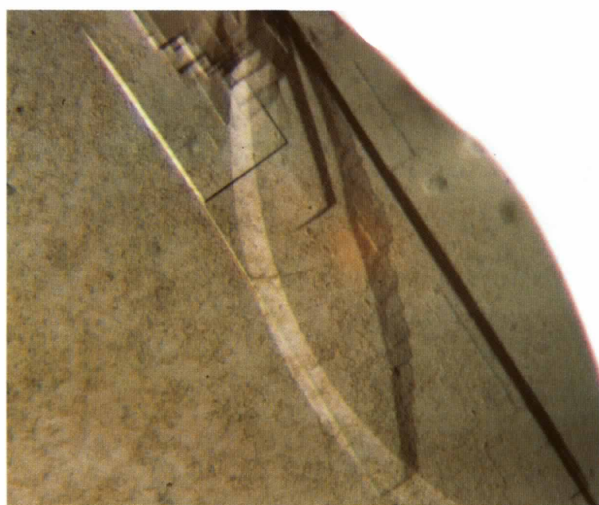
(a)

(b)

Fig. 2. Crystals grown from different conditions of 3,6-diketocamphane monooxygenase oxygenating subunits from *P. putida*. (a) Condition 1, thin stacked crystalline plates. (b) Condition 2, thicker rod-shaped crystals.

Table 1. *Statistics for the data collected from the ammonium sulfate grown crystals (condition 2)*

Wavelength (Å)	1.00
Resolution (Å)	18–2.5
Number of measured reflections	94431
Number of unique reflections	27982
Completeness (all data) (%)	97.3
Completeness (highest-resolution shell) (%)	96.9
$I > 2\sigma(I)$ (%)	80.6
R_{sym}† (all data) (%)	7.8
R_{sym}† (highest resolution shell) (%)	40.7
$R_{stand}(I)$‡ (all data) (%)	6.1

† $R_{sym} = \sum(\sum_i |I_i - \langle I \rangle|)/ \sum \langle I \rangle$ where $\langle I \rangle$ is the mean of the intensity measurements, I_i, and the summation extends over all reflections. ‡ $R_{stand}(I) = \langle \sigma(I) \rangle / \langle I \rangle$.

ammonium sulfate. These crystals diffracted to beyond 2 Å resolution, allowing a complete data set to be collected to 2.5 Å resolution at 100 K. The space group was again identified as $P2_12_12_1$, with unit-cell dimensions $a = 54.6$, $b = 93.2$ and $c = 154.1$ Å. The statistics for the 2.5 Å data are given in Table 1. The asymmetric unit could contain either two subunits with a calculated solvent content of 47%, or one subunit with a solvent content of 74% (Matthews, 1968). Provided the molecule retains its dimeric state in the crystal, the asymmetric unit is unlikely to contain a single subunit as there are no proper rotational axes in $P2_12_12_1$. One subunit per asymmetric unit cannot be ruled out completely, as the protein could crystallize as a monomer rather than in its dimeric form.

Self-rotation functions (Collaborative Computational Project, Number 4, 1994) calculated with a range of integration radii and with different resolution limits showed no particular features for a range of values for κ. This could favour one molecule per asymmetric unit, but even in the case of two molecules per asymmetric unit the self-rotation function could be obscured by the relatively strong anisotropy of the data – the ratio of eigenvalues of the anisotropic distribution of the structure factors is 0.59:0.50:1.00 as estimated by the program *SFCHECK* (A. Vagin, personal communication). Alternatively, the non-crystallographic molecular dyad could be almost parallel to one of the crystallographic axes and thus be shadowed by the latter in the self-rotation function. The native Patterson does not contain any significant features which could indicate pseudotranslation. Therefore, if there is a molecular dyad in these crystals it is unlikely that it is precisely parallel to one of the crystallographic twofold axes.

The observation that a similar crystal form can be grown from both PEG 8000 and ammonium sulfate will be useful when carrying out substrate soaks or co-crystallization experiments where the transfer of crystals from ammonium sulfate into polyethylene glycol may be necessary. A search for heavy-atom derivatives is currently in progress.

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