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Crystallization and X-ray diffraction of a halogenating enzyme, tryptophan 7-halogenase, from *Pseudomonas fluorescens*

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Chlorination of natural products is often required for their biological activity; notable examples include vancomycin, the last-ditch antibiotic. It is now known that many chlorinated natural products are made not by haloperoxidases, but by $FADH_2$-dependent halogenases. The mechanism of the flavin-containing enzymes is obscure and there are no structural data. Here, crystals of PrnA (tryptophan 7-halogenase), an enzyme that regioselectively chlorinates tryptophan, cocrystallized with tryptophan and FAD are reported. The crystals belong to the tetragonal space group $P4_32_12$ or $P4_12_12$, with unit-cell parameters $a = b = 67.8$, $c = 276.9$ Å. A data set to 1.8 Å with 93% completeness and an $R_{\rm merge}$ of 7.1% has been collected from a single flash-cooled crystal. A method for incorporating selenomethionine in a *Pseudomonas fluorescens* expression system also is reported.

1. Introduction

Organohalogen compounds are found in bacteria, fungi, plants and animals. More than 3800 halometabolites have been found so far, of which over 3000 are either chlorinated or brominated (fluorinated and iodinated compounds are rare; Gribble, 2003). The increase in the number of known compounds from a mere 200 in 1971 mirrors the general revitalization of interest in natural products as a potential source of new medicinal drugs. Many of these chlorinated natural products are biologically active, ranging from vancomycin, which depends on chlorine for its full bactericidal activity (Williams & Bardsley, 1999), to the potent anticancer compound cryptophycin A (Sailler & Glombitza, 1999). For about 35 years, haloperoxidases were thought to catalyse all halogenation reactions. However, elucidation of the reaction mechanism of haloperoxidases showed that these enzymes produce free hypohalous acids (Messerschmidt *et al.*, 1997; Sundaramoorthy *et al.*, 1998), which are the actual halogenating agents, and thus halogenation 'catalysed' by haloperoxidases proceeds without substrate specificity and regioselectivity. From a biochemical viewpoint, the lack of substrate specificity and regioselectivity of halogenation reactions catalysed by haloperoxidases made it very unlikely that these enzymes could be involved in complex biosynthetic pathways for halometabolite formation.

Dairi *et al.* (1995) identified the gene coding for the halogenating enzyme involved in chlorotetracycline biosynthesis. This gene and the protein derived from the DNA sequence showed no similarity to haloperoxidases or perhydrolases. This was the first evidence for a different type of halogenating enzyme. During investigations of the biosynthesis of the antifungal antibiotic pyrrolnitrin by *Pseudomonas fluorescens* (Fig. 1), two genes were detected that coded for two halogenating enzymes with different substrate specificities (Hammer *et al.*, 1997; Keller *et al.*, 2000). Both these enzymes require $FADH_2$, oxygen, Cl^- or Br^- for activity (Hammer *et al.*, 1997; Keller *et al.*, 2000). Pyrrolnitrin is active against *Rhizoctonia* spp., *Fusarium* spp. and other plant pathogenic fungi and has been used as a lead structure in the development of a new phenylpyrrole agricultural fungicide. Pyrrolnitrin is made by four enzymatic steps and the genes for all four enzymes are found in a number of *Pseudomonas* spp. (Hammer *et al.*, 1999; Ligon *et al.*, 2000). Members of this superfamily, the $FADH_2$-dependent halogenases, have been shown to be involved in the synthesis of the antibiotics balhimycin (Puk *et al.*, 2002) and vancomycin (Williams & Bardsley, 1999). Tryptophan 7-halogenase (PrnA) catalyses the regioselective chlorination and bromination of tryptophan in the 7-position of the indole-ring system. The third enzyme in the pyrrolnitrin biosynthetic pathway catalyses the regioselective chlorination of monodechloroaminopyrrolnitrin at the 3-position of the pyrrole ring and was named monodechloroaminopyrrolnitrin 3-halogenase (PrnC; Hohaus *et al.*, 1997). The mechanism of chlorination remains unclear and the absence of structural data has hindered progress towards mechanistic understanding. Here, we report the purification and crystallization of

tryptophan 7-halogenase from *P. fluorescens*. Diffraction data were collected from a single flash-cooled crystal to 1.8 Å resolution. The data obtained are an important step towards elucidation of the reaction mechanism.

2. Materials and methods

2.1. Protein isolation and purification

Growth of bacteria and protein purification were carried out according to the published method (Keller *et al.*, 2000). The plasmid pPEH14(prnA) encodes for expression of untagged full-length PrnA (1–538; GenBank AAB97504; Hammer *et al.*, 1997). Details of the expression plasmid for *P. fluorescens* have been described (Kirner *et al.*, 1998). Transformed *P. fluorescens* BL915 ΔORF1-4 with plasmid pPEH14(prnA) was inoculated in a corn-molasses medium and was cultured for 3 d at 303 K. Cells were harvested by centrifugation and disrupted by sonication in 100 m*M* phosphate buffer pH 7.2 containing leupeptin, pepstatin, aprotinin and phenylmethylsulfonyl fluoride (Roche) as protease inhibitors. The cell debris was removed by centrifugation and the cell extract was dialysed against 10 m*M* phosphate buffer pH 7.2 at 277 K. The sample was loaded on a Q-Sepharose FF column (Amersham Biosciences) and protein was eluted using a linear-gradient elution from 0 to 1 *M* NaCl at room temperature. Fractions containing PrnA were pooled. Ammonium sulfate was added to a final concentration of 1 *M* and the protein solution was then loaded onto a butyl Sepharose FF column (Amersham Biosciences). PrnA was eluted using a linear gradient from 1 to 0 *M* ammonium sulfate, again at room temperature. The protein was further purified on a Superdex-200 column (Amersham Biosciences) at room temperature and eluted with a molecular weight consistent with a dimer (120 kDa). PrnA was loaded onto a SDS–PAGE gel and showed a single band. The protein solution was dialysed against 20 m*M* HEPES buffer pH 7.2 and 0.1 *M* NaCl and concentrated to 8 mg ml^{-1} for crystallization. A yield of 5 mg of protein per litre of culture broth was obtained. Mass spectroscopy showed a molecular weight of 61 074 Da, matching the sequence prediction. The protein was confirmed as authentic by mass spectroscopy with trypsin digestion and an enzyme-activity assay (Keller *et al.*, 2000).

2.2. Crystallization and data collection

Prior to crystallization, the protein was incubated with 5 m*M* FAD, 5 m*M* DTT and 20 m*M* L-tryptophan for 1 h at room temperature. Protein crystallization was carried out by sitting-drop vapour diffusion at 293 K. Each well contained 2 µl protein solution and 2 µl precipitant solution with 100 µl precipitant solution in the reservoir. The crystals were obtained from a condition consisting of 10%(*v*/*v*) PEG 8000, 0.1 *M* imidazole buffer pH 8.0 and 0.2 *M* $Ca(CH_3CO_2)_2$. The crystals are yellow, indicating that FAD was bound to the protein. The crystals are diamond-shaped, with dimensions of 0.6 × 0.25 × 0.25 mm, and are shown in Fig. 2. A crystal was flash-cooled in a cryoprotectant consisting of 10%(*v*/*v*) PEG 8000, 20 m*M* L-tryptophan, 20%(*v*/*v*) glycerol, 0.2 *M* $Ca(CH_3CO_2)_2$ and 0.1 *M* imidazole buffer pH 8.0 at 100 K. Data to 1.8 Å were collected at the ESRF beamline ID-14-1 using an ADSC Q4 CCD detector. 450 frames were collected, with 0.5° oscillation and 5 s exposure time per frame. The data were indexed and integrated using *MOSFLM* (Collaborative Computational Project, Number 4, 1994; Leslie, 1992) and the data were scaled using *SCALA* (Collaborative Computational Project, Number 4, 1994).

2.3. Incorporation of selenomethionine into the protein

We are planning to determine the structure using the multiwavelength anomalous diffraction (MAD) method. However, no approach has been reported concerning selenomethionine incorporation using a *P. fluorescens* expression system. In *Escherichia coli* expression systems, the inhibition of methionine biosynthesis pathway is a very popular approach to incorporate selenomethionine (Doublié, 1997). This method was applied to the *P. fluorescens* system in order to obtain selenomethionine-labelled protein. The transformed cells were inoculated in a minimal medium containing 0.8%(*w*/*v*) K_2HPO_4, 0.3%(*w*/*v*) KH_2PO_4, 0.1% $(NH_4)_2SO_4$, 0.675%(*w*/*v*) sodium succinate and 0.0072%(*w*/*v*) $MgSO_4$ pH 7.0 (Kirner *et al.*, 1996). Lysine, phenylalanine, threonine, isoleucine, leucine, valine and selenomethionine were added as the free acids to the minimal medium to a final concentration of 100 mg l^{-1} before inoculation. Kanamycin and tetracycline were added to the culture to final concentrations of 50 and 30 mg l^{-1}, respectively. The methods for the cell culture and the protein purification were the same as that described above for the native protein, except that DTT was present during all protein purification steps. A yield of 2 mg selenomethionine-labelled protein per litre of culture was obtained. This result is consistent with the lower biomass and slower growth of bacteria under these conditions.

Table 1
Summary of data-collection statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	0.933
Resolution (Å)	54.66–1.80 (1.90–1.80)
Space group	$P4_32_12$ or $P4_12_12$
Unit-cell parameters (Å, °)	$a = b = 67.83$, $c = 276.91$, $\alpha = \beta = \gamma = 90$
No. unique reflections	57128
Multiplicity	9.1 (5.2)
$I/\sigma(I)$	6.4 (2.3)
Completeness (%)	93 (68)
$R_{\rm merge}$† (%)	7.1 (33.1)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i - \langle I\rangle| / \sum_{hkl}\sum_i \langle I\rangle$, where I_i is the intensity of the ith measurement of a reflection with indices hkl and $\langle I\rangle$ is the weighted mean of the reflection intensity.

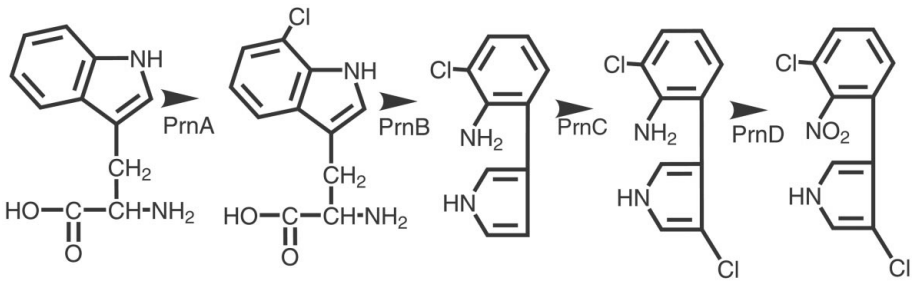

Figure 1
Pyrrolnitrin biosynthetic pathway.

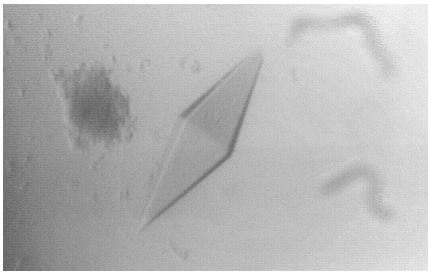

Figure 2
A crystal (0.6 × 0.25 × 0.25 mm) of tryptophan 7-halogenase (PrnA).

3. Results and discussion

The data suggest that the crystal is primitive tetragonal. Data reduction suggested Laue group $P422$ and examination of the systematic absences indicated that the crystal belongs to space group $P4_32_12$ or $P4_12_12$. The unit-cell parameters are $a = b = 67.8$, $c = 276.9$ Å, $\alpha = \beta = \gamma = 90^\circ$. Because of the marked asymmetry in the crystal cell edges, correct orientation of the crystal in the loop was vital to avoid overlapping spots. Full details of the X-ray data are listed in Table 1. Calculations suggest that there is a monomer in the asymmetric unit (Matthews coefficient of 2.6 $Å^3$ Da^{-1}; 52.3% solvent).

Mass spectroscopy showed that the native protein and for the selenomethionine-labelled protein had molecular weights of 61 074 and 61 641 Da, respectively. This result indicates that all 12 methionine residues of this protein were successfully replaced by selenomethionine. Crystals of the selenomethionine-labelled protein have been obtained under the same conditions as normal protein and appear to have similar diffraction properties. The structure will be determined using the MAD method.

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