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Overexpression, purification and crystallization of PhzA, the first enzyme of the phenazine biosynthesis pathway of *Pseudomonas fluorescens* 2-79

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Phenazines are broad-spectrum antibiotic metabolites produced by organisms such as *Pseudomonas* and *Streptomyces*. Phenazines have been shown to enhance microbial competitiveness and the pathogenic potential of the organisms that synthesize them. PhzA (163 residues, approximate molecular weight 18.7 kDa) is the product of the first of seven genes of the operon responsible for phenazine biosynthesis in *P. fluorescens* 2-79. This enzyme is thought to catalyse one of the final steps in the formation of phenazine-1-carboxylic acid, the end product of phenazine biosynthesis in *P. fluorescens* 2–79. Here, the purification and crystallization of recombinant PhzA are reported. Crystals diffracting to 2.1 Å were obtained using 1.6 *M* magnesium sulfate and 2-morpholinoethanesulfonic acid monohydrate (MES) buffer pH 5.2–5.6. Crystals of both native and seleno-L-methionine-labelled protein belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 66.8, b = 75.3, c = 84.5 Å. The asymmetric unit contains one dimer of PhzA.

1. Introduction

Phenazine biosynthesis, an offshoot of the shikimic acid pathway, results in the production of phenazine-1-carboxylic acid (PCA), pyocyanin and other compounds of the phenazine family. Phenazines are broad-spectrum antibiotic metabolites synthesized by organisms such as *Pseudomonas*, *Streptomyces*, *Erwinia* and *Burkholderia* (Turner & Messenger, 1986). Phenazines participate in redox reactions and produce superoxide, hydroxyl and peroxyl radicals. These radicals are lethal to fungi and bacteria (both Gram-negative and Gram-positive) and generate pathogenic symptoms in higher organisms. Pyocyanin, the end product of phenazine biosynthesis in *P. aeruginosa*, has been shown to be responsible for the disruption of human neutrophils (Usher *et al.*, 2002) and the effective killing of nematodes (Mahajan-Miklos *et al.*, 1999), while phenazine-1-carboxylic acid (PCA) produced by the biological control agent *P. fluorescens* 2-79 is responsible for the suppression of soil-borne plant pathogens (Thomashow & Weller, 1988; Pierson & Thomashow, 1992).

A seven-gene operon is responsible for phenazine biosynthesis in *P. chlororaphis* (Chin-A-Woeng *et al.*, 2001), *P. aureofaciens* (Pierson *et al.*, 1995), *P. fluorescens* (Mavrodi *et al.*, 1998) and *P. aeruginosa* (Mavrodi *et al.*, 2001). This operon encodes seven enzymes, generally named PhzA–G, that are capable of converting the precursor of phenazine biosynthesis, chorismic acid, to phenazine-1-carboxylic acid. PCA, the end product of phenazine biosynthesis in *P. fluorescens* 2-79, can be converted to a range of phenazine compounds in other pseudomonads. Three of the seven biosynthetic enzymes, PhzC, PhzD and PhzE, are similar to enzymes of shikimic acid metabolism. The structure of PhzD from the phenazine biosynthesis operon of *P. aeruginosa* has recently been described in the literature (Parsons *et al.*, 2003). Structural information about other Phz enzymes is not yet available, although some clues about their function have been obtained by gene-expression studies (McDonald *et al.*, 2001). These studies have shown that PhzF and PhzG are absolutely essential, while PhzA and PhzB are involved in the final stages of phenazine biosynthesis, particularly in steps successive to the formation of *trans*-2,3-dihydro-3-hydroxyanthranilic acid (DHHA; Fig. 1). In the absence of other enzymes of the pathway, PhzA and PhzB alone are incapable of synthesizing PCA; however, their absence leads to an eightfold decrease in the efficiency with which PhzC–G produce PCA. The enzymes PhzA and PhzB, which have a high degree of sequence similarity with each other, have no sequence relatives in the Protein Data Bank (PDB; Berman *et al.*, 2000) or similarity to other proteins of known function.

PhzA from the phenazine biosynthesis pathway of *P. fluorescens* 2-79 is a 163-amino-acid protein (approximate molecular weight 18.7 kDa) and has over 90% sequence identity

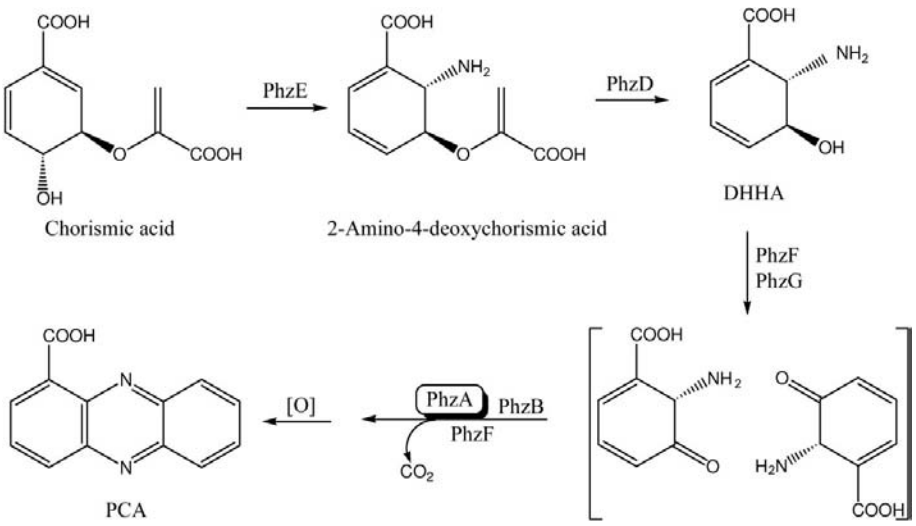

Figure 1
Phenazine-1-carboxylic acid biosynthesis pathway of *P. fluorescens* (figure modified after McDonald *et al.*, 2001). DHHA, *trans*-2,3-dihydro-3-hydroxyanthranilic acid; PCA, phenazine-1-carboxylic acid.

with PhzB of the same operon and over 80% sequence identity with similar genes in *P. chlororaphis*, *P. aeruginosa etc*. Fold-recognition studies using *3D-PSSM* (Kelley *et al.*, 2000) and other structure-prediction programmes indicate the nearest structural homologue of PhzA to be Δ^5-3-ketosteroid isomerase from *P. putida*. However, the active site of PhzA cannot be established with confidence from this alone; hence, we have initiated this study for the elucidation of the structure and function of PhzA.

We describe here the details of over-expression, purification and crystallization of recombinant PhzA together with details of the first diffraction data collected from native and seleno-L-methionine-substituted crystals.

2. Expression and purification

The oligonucleotide primers PhzA-up (5′-ACT GCA TAT GCC CGG TTC G-3′) and PhzA-low (5′-CTA GGA TCC ATG TTC AAT CTC CAA T-3′) were used to generate a 534 bp DNA fragment encoding PhzA from *P. fluorescens* 2-79 by PCR. This fragment was amplified with a PTC-200 thermal cycler (MJ Research). PCR products were digested with *Nde*I and *Bam*HI, gel-purified, cloned behind a T7 promoter in the N-terminal His-tag fusion vector pET-15b (Novagen) and single-pass sequenced to confirm the integrity of the resultant fusion.

Escherichia coli Rossetta (DE3)/pLysS (Novagen) cells containing pET-15b-PhzA were grown in Terrific Broth medium supplemented with 100 µg ml^{-1} ampicillin and 34 µg ml^{-1} chloramphenicol at 310 K to an OD_{600} of 0.6. Expression of PhzA was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 m*M*. For greater solubility of the resultant protein, the induced cells were grown overnight at 292 K. After harvesting by centrifugation (6000*g*, 25 min, 277 K), the cells were resuspended in ice-cold 50 m*M* sodium phosphate pH 8.0 and 300 m*M* NaCl buffer containing 5 m*M* PMSF. The cells were then disrupted in a microfluidizer.

The soluble lysis fraction was cleared by ultracentrifugation (150 000*g*, 45 min, 277 K) and loaded onto a nickel-chelation column (Ni–NTA, Qiagen) equilibrated in buffer containing 50 m*M* sodium phosphate pH 8.0 and 300 m*M* NaCl. After washing, the bound protein was eluted with a 10–500 m*M* imidazole gradient. PhzA elutes at a concentration of 330 m*M* imidazole. Fractions containing PhzA (identified by analysis on 15% SDS–PAGE) were pooled and dialysed overnight in 20 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl buffer (gel-filtration buffer) to remove imidazole. Thrombin (one unit per 20 mg of protein; bovine thrombin, Sigma) cleavage was undertaken at room temperature for 2.5 h and thrombin removed by passing the protein over a benzamidine column (Amersham Biosciences). The protein was concentrated (Amicon concentrators) and loaded onto a Superdex 75 (Amersham Biosciences) gel-filtration column equilibrated in gel-filtration buffer. PhzA elutes as a dimer from the gel-filtration column. This two-step purification procedure yielded 100 mg of pure protein suitable for crystallization trials from 2 l of shaken culture. PhzA was concentrated to 20–30 mg ml^{-1} in 20 m*M* Tris–HCl pH 8.0 and 150 m*M* NaCl and stored as frozen aliquots at 193 K.

To facilitate experimental phasing using anomalous dispersion methods, seleno-L-methionine labelling of PhzA was initiated by inhibition of methionine biosynthesis (Doublié, 1997) in Rossetta (DE3)/pLysS *E. coli* cells expressing pET15b-PhzA. These cells were grown overnight in Luria–Bertani media supplemented with 100 µg ml^{-1} ampicillin and 34 µg ml^{-1} chloramphenicol, pelleted and resuspended in M9 media containing 50 µg ml^{-1} ampicillin and 17 µg ml^{-1} chloramphenicol. This culture was grown at 310 K to mid-log phase (OD_{600} of 0.8) and the amino acids lysine, phenylalanine and threonine were then added to 100 mg l^{-1} and isoleucine, leucine and valine to 50 mg l^{-1}, with the final addition of 60 mg l^{-1} seleno-L-methionine. This culture was allowed to cool to 292 K before overnight induction with 1 m*M* IPTG. The purification protocol for the labelled protein was identical to that of the native protein. Substitution of the methionine residues by selenomethionines was checked by MALDI–MS analysis. The (SeMet)-PhzA molecular weight (19 114 Da) corresponds to the introduction of three Se atoms, indicating full substitution, since PhzA contains methionine residues at positions 1, 28 and 141. The MALDI–MS-verified weight of PhzA is 18 973 Da.

3. Crystallization

Crystallization was initiated at 292 K using the hanging-drop vapour-diffusion method with Crystal Screen and Crystal Screen 2 from Hampton Research (Jancarik & Kim, 1991). 15, 20 and 25 mg ml^{-1} concentrations of protein were used for initial screening, with 1 µl of protein mixed with a similar amount of precipitant solution. Large single crystals (0.2 × 0.2 × 0.6 mm) were obtained after several days in 0.8 *M* sodium/potassium tartrate and 0.1 *M* sodium HEPES pH 7.5. They were tested at the European Synchrotron Radiation Facility (ESRF,

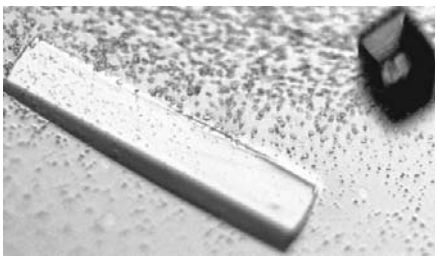

Figure 2
A native crystal of PhzA (approximate dimensions 0.3 × 0.3 × 0.5 mm).

Table 1
Data-collection statistics.

Values in parentheses correspond to the highest resolution shell.

Data collection	Long axis	Native	SeMet peak	SeMet inflection
Wavelength (Å)	0.934 (ID14-EH1)	0.934 (ID14-EH1)	0.9792 (BM14)	0.9797 (BM14)
Resolution (Å)	20.0–6.5 (6.6–6.5)	20.0–2.1 (2.2–2.1)	20.0–3.5 (3.6–3.5)	20.0–3.5 (3.6–3.5)
Space group	*R*32	$P2_12_12_1$	$P2_12_12_1$	
Unit-cell parameters (Å, °)	$a = b = 95.4$, $c = 1955.7$, $\alpha = \beta = 90$, $\gamma = 120$	$a = 66.8$, $b = 75.2$, $c = 84.5$, $\alpha = \beta = \gamma = 90$	$a = 67.3$, $b = 75.4$, $c = 84.6$, $\alpha = \beta = \gamma = 90$	
V_M (Å^3 Da^{-1})	—	2.5	2.5	
Total measurements	20396 (992)	102278 (13252)	27152 (2202)	27220 (2246)
Unique reflections	7142 (331)	47100 (6080)	10461 (856)	10487 (873)
Average redundancy	2.8 (3.0)	2.9 (2.2)	2.9 (2.6)	2.6 (2.6)
$I/\sigma(I)$	7.1 (2.8)	10.7 (2.6)	19.7 (4.8)	9.5 (4.7)
Completeness (%)	94.3 (100)	98.2 (97.7)	99.8 (100)	99.7 (100)
Anomalous completeness† (%)	—	—	98.3 (100)	99.8 (100)
Wilson B (Å^2)	—	44	30	33
R_{sym}‡	10.5 (39.8)	4.6 (36.8)	9.3 (19.6)	8.3 (14.6)
f'/f''	—	—	−6.5/4.7	−9.6/0.7

† Completeness calculations treat Friedel pairs as separate observations. ‡ $R_{sym} = \sum I(h)_j - \langle I(h)\rangle|/\sum I(h)_j$, where $I(h)_j$ is the scaled observed intensity of the ith symmetry-related observation of reflection h and $\langle I(h)_i\rangle$ is the mean value.

Grenoble, France; beamline ID14-EH1) for diffraction quality. These crystals, belonging to space group *R*32, had an extremely long *c* axis ($a = b = 95.4$, $c = 1955.7$ Å, $\alpha = \beta = 90$, $\gamma = 120°$), making them unsuitable for data collection. A second crystal form appeared with 1 µl 26 mg ml^{-1} protein solution and an equal volume of 1.6 *M* magnesium sulfate and 0.1 *M* MES in the pH range 5.2–5.6. The diffraction properties of these crystals, which had dimensions 0.3 × 0.3 × 0.9 mm (Fig. 2), were much more suitable than the previous ones; these crystals were used for data collection.

4. Data collection

The crystals were cryoprotected by soaking briefly in mother liquor supplemented with 10%(*w*/*v*) sucrose and 10%(*w*/*v*) xylitol before flash-freezing in liquid nitrogen. A complete data set was obtained to a resolution of 2.1 Å for native crystals (beamline ID-14 EH1, ESRF). As molecular replacement using structures predicted to be similar to PhzA (Δ^5-3-ketosteroid isomerase; representative PDB code 1oh0; Kim *et al.*, 1997; nuclear transport factor 2; representative PDB code 1gy6; Bayliss *et al.*, 2002) was not successful, MAD data for wavelengths corresponding to both peak and inflection were collected to 3.5 Å for selenomethionine-labelled crystals at beamline BM-14 of the same synchrotron. The *XDS* package (Kabsch, 1993) was used to index, integrate and scale the collected data. The crystals belong to space group $P2_12_12_1$ and have unit-cell parameters $a = 66.8$, $b = 75.3$, $c = 84.5$ Å (Table 1). Assuming the presence of one dimer in the asymmetric unit, the solvent content of the crystals is 57%, corresponding to a Matthews coefficient (Matthews, 1968) V_M of 2.9 Å^3 Da^{-1}. We are currently using these data to derive initial phases.

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