The purification, crystallization and preliminary structural characterization of FAD-dependent monooxygenase PhzS, a phenazine-modifying enzyme from *Pseudomonas aeruginosa*

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The blue chloroform-soluble bacterial metabolite pyocyanin (1-hydroxy-5-methyl-phenazine) contributes to the survival and virulence of *Pseudomonas aeruginosa*, an important Gram-negative opportunistic pathogen of humans and animals. Little is known about the two enzymes, designated PhzM and PhzS, that function in the synthesis of pyocyanin from phenazine-1-carboxylic acid. In this study, the FAD-dependent monooxygenase PhzS was purified and crystallized from lithium sulfate/ammonium sulfate/sodium citrate pH 5.5. Native crystals belong to space group $C2$, with unit-cell parameters $a = 144.2$, $b = 96.2$, $c = 71.7$ Å, $\alpha = \gamma = 90$, $\beta = 110.5°$. They contain two monomers of PhzS in the asymmetric unit and diffract to a resolution of 2.4 Å. Seleno-L-methionine-labelled PhzS also crystallizes in space group $C2$, but the unit-cell parameters change to $a = 70.6$, $b = 76.2$, $c = 80.2$ Å, $\alpha = \gamma = 90$, $\beta = 110.5°$ and the diffraction limit is 2.7 Å.

1. Introduction

The phenazine compounds produced by fluorescent *Pseudomonas* species are biologically active metabolites that function in microbial competitiveness, the suppression of soil-borne plant pathogens and virulence in human and animal hosts (Mavrodi *et al.*, 2006; Price-Whelan *et al.*, 2006). The ubiquitous soil bacterium *P. aeruginosa* is the most extensively studied phenazine-producing fluorescent pseudomonad because of its environmental versatility, its resistance to antibiotics and its ability to cause disease in hosts ranging from animals to plants, insects and nematodes. In humans, *P. aeruginosa* is an opportunistic pathogen of immunocompromized, burned or injured patients and can cause both acute and chronic lung disease. The bacterium produces a variety of redox-active phenazines, including the distinctive blue pigment pyocyanin (1-hydroxy-5-*N*-methyl-phenazine), 1-hydroxyphenazine, phenazine-1-carbox-amide and the precursor of all of these compounds, phenazine-1-carboxylic acid (PCA; Mavrodi *et al.*, 2006). *P. aeruginosa* is the only known producer of pyocyanin (Reyes *et al.*, 1981), which is likely to have been responsible for the blue–green colour of infected wounds described in the literature as early as the 1850s (Fordos, 1859).

Pyocyanin is a virulence factor toxic to eukaryotic cells. It has been shown to inhibit mammalian cell respiration (Stewart-Tull & Armstrong, 1972), disrupt the beating of human cilia (Wilson *et al.*, 1987) and inhibit lymphocyte proliferation and epidermal cell growth (Sorensen *et al.*, 1983). It also interferes with calcium homeostasis, triggers apoptosis in neutrophiles, causes imbalance of protease–antiprotease activity in the airways of cystic fibrosis patients (Britigan *et al.*, 1999) and plays a role in pulmonary tissue damage observed with chronic lung infections (Wilson *et al.*, 1988). *P. aeruginosa* strains with defective pyocyanin biosynthesis are less virulent and more susceptible to the immune response in the lung-infection mouse model (Lau *et al.*, 2004).

The conversion of phenazine-1-carboxylic acid to pyocyanin is thought to occur in two steps. PhzM, an *S*-adenosyl-methionine-dependent methyltransferase, first converts PCA to 5-methylphen-azine-1-carboxylic acid betaine, which then undergoes hydroxylative decarboxylation catalyzed by PhzS, an FAD-dependent mono-oxygenase, to yield pyocyanin (Fig. 1; Mavrodi *et al.*, 2001). Whilst the
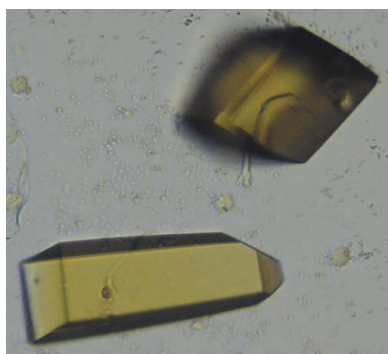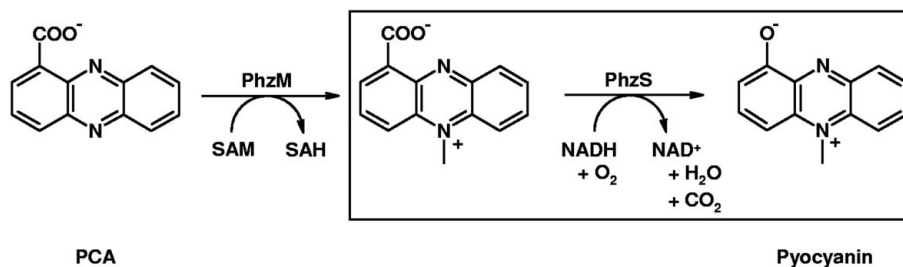
Figure 1
Proposed pathway for the synthesis of pyocyanin.

role of pyocyanin in *P. aeruginosa* infections is well established, little is known about the enzymes involved in its biosynthesis. We have therefore initiated structural and functional studies of PhzS and PhzM, which are potential targets for inhibition of pyocyanin biosynthesis in patients infected by *P. aeruginosa*.

PhzS from *P. aeruginosa* is a 402-residue dimeric protein with a monomer molecular weight of 43.6 kDa. Predictions from the fold-recognition program 3*DPSSM* (Kelley *et al.*, 2000) indicate that it possesses significant fold similarity to FAD-dependent oxido-reductases and indicate a 17% sequence similarity to *p*-hydroxy-benzoate hydroxylase (PDB code 1phh; Schreuder *et al.*, 1988), a flavoprotein involved in the degradation of aromatic compounds and a model for enzymes involved in the oxygenation of a substrate.

Both PhzS and *p*-hydroxybenzoate hydroxylase belong to the class of FAD-dependent monooxygenases. These enzymes catalyse the insertion of one atom of dioxygen into a variety of substrates while the other O atom is reduced to water. The monooxygenases use an external reducing agent to provide electrons to reduce oxygen to hydrogen peroxide, which is then responsible for the insertion of oxygen into the substrate. These enzymes are reduced by NAD(P)H to give $FADH_2$, which then binds molecular oxygen to form a spec-troscopically characterized highly reactive peroxide-linked flavin. This intermediate is decomposed by the nucleophilic attack of an adjacent substrate, resulting in O-atom transfer (Dong *et al.*, 2005). In PhzS, this leads to a decarboxylation of the substrate with concomi-tant incorporation of a hydroxyl group.

Here, we report the large-scale expression of recombinant PhzS and the first diffraction data collected from native and seleno-L-methionine-labelled protein crystals as an initial step towards understanding the reaction mediated by PhzS and its substrate specificity.

2. PhzS overexpression and purification

The oligonucleotide primers PhzS-up (5′-TATATACATATGAGC-GAACCCATCGAT-3′) and PhzS-low (5′-TTTTTTGGATCCTAG-CGTGGCCGTTC-3′) were used to generate a 1218 bp DNA fragment encoding PhzS from *P. aeruginosa* PA01 by PCR. This fragment was amplified using a PTC-200 thermocycler (MJ Research). The PCR product was 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.

The *phzS* gene was expressed in *Escherichia coli* Rosetta pLysS (Novagen). An overnight culture from a single colony was diluted into fresh Terrific Broth (TB) supplemented with 100 mg l^{-1} ampi-cillin and 34 mg l^{-1} chloramphenicol. The cells were grown at 310 K in TB medium with vigorous shaking to $OD_{600} = 0.6$. Gene expression was induced with 1 m*M* isopropyl β-D-thiogalactoside (IPTG) for 3 h

at 310 K. The cells were harvested by centrifugation for 15 min at 6000*g*, resuspended in 50 m*M* Na_2HPO_4 pH 8.0, 300 m*M* NaCl supplemented with 2 m*M* PMSF and then lysed with a fluidizer. The lysate was then centrifuged at 150 000*g* (Beckmann Optima L-70K ultracentrifuge; Ti-45 rotor) for 30 min and the resulting supernatant filtered, adsorbed and eluted from Ni–NTA affinity resin. Fractions containing PhzS, as judged by sodium dodecyl sulfate (SDS) gel electrophoresis, were pooled, dialyzed against 50 m*M* Tris pH 8.0 and 150 m*M* NaCl and concentrated prior to purification using gel filtration on Superdex 200 (GE Healthcare) in the same buffer. The purified protein was then concentrated to 20 mg ml^{-1}. In pET-15b, the N-terminal His_6 tag can be removed with thrombin. However, the cleavage site proved to be inaccessible and the PhzS fusion protein was purified in the uncleaved form.

(*a*)

(*b*)
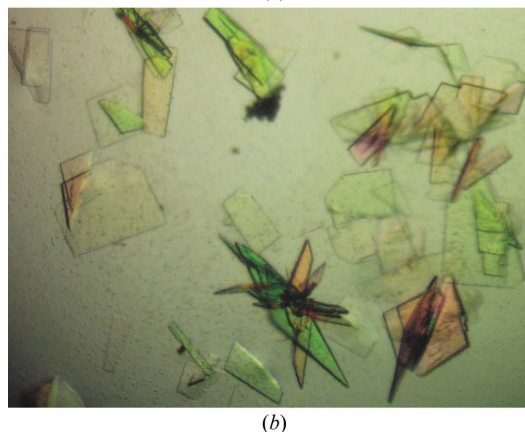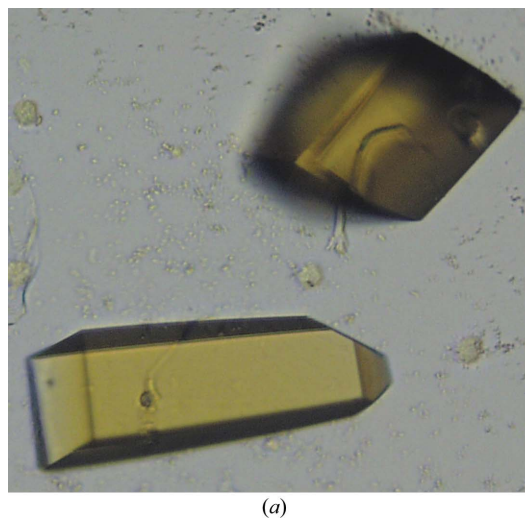
Figure 2
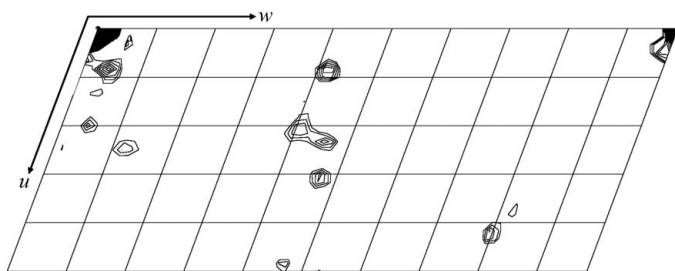(*a*) Native and (*b*) seleno-L-methionine-labelled crystals of PhzS.

Seleno-L-methionine labelling was achieved by inhibition of methionine biosynthesis (Doublié, 1997). *E. coli* BL21 pLysS cells bearing pET-15b with His$_6$-tagged PhzS were grown overnight in Luria–Bertani broth supplemented with ampicillin and chloramphenicol, pelleted and suspended in M9 broth amended with 50 mg ml^{-1} ampicillin and 17 mg ml^{-1} chloramphenicol. The culture was grown at 310 K to mid-log phase (OD$_{600}$ = 0.8) and amino acids (lysine, phenylalanine and threonine to 100 mg l^{-1}; isoleucine, leucine and valine to 50 mg l^{-1}) were added. The culture was supplemented with 60 mg l^{-1} seleno-L-methionine and allowed to cool to 293 K before overnight induction with 1 mM IPTG. The labelled protein showed purification properties identical to those of the native enzyme.

3. PhzS crystallization

Initial crystallization conditions were determined at room temperature with Crystal Screen and Crystal Screen 2 from Hampton Research (Cudney *et al.*, 1994; Jancarik & Kim, 1991). The protein concentration was screened at 10 and 15 mg ml^{-1} by dilution of the protein stock with gel-filtration buffer. The hanging-drop vapour-diffusion method with drops consisting of 1.2 μl protein and 1.2 μl precipitant solution equilibrated against 500 μl reservoir volume at room temperature was used throughout. Conditions that produced microcrystalline precipitates were optimized with respect to protein concentration, precipitant concentration and pH to reduce the amount of nucleation and to increase the size and appearance of the crystals obtained. Diffraction-quality crystals were obtained at 12 mg ml^{-1} PhzS concentration and a reservoir containing 0.7–1.0 M lithium sulfate, 0.1 M sodium citrate pH 5.0–5.5, 0.5 M ammonium sulfate. The crystals are yellow owing to the presence of FAD and grow to dimensions of 0.3 × 0.3 × 0.2 mm (Fig. 2*a*) in approximately 3 d. Seleno-L-methionine-labelled crystals of PhzS (Fig. 2*b*) grow as thin fragile plates and were obtained under two different conditions consisting of 14–17%(*w*/*v*) PEG 10 000, 0.1 M ammonium acetate, 0.1 M Bis-Tris pH 5.5 and 23–26%(*w*/*v*) PEG 3350, 0.1 M Bis-Tris pH 5.8–6.0, 0.2 M ammonium sulfate.

4. Data collection

To achieve cryoprotection prior to data collection, PhzS crystals were washed briefly in mother liquor supplemented with 10%(*w*/*v*) sucrose and 10%(*w*/*v*) xylitol and then flash-cooled in liquid nitrogen. Crystals of the native protein belong to space group *C*2, with unit-cell parameters *a* = 144.2, *b* = 96.2, *c* = 71.7 Å, α = γ = 90, β = 110.5°, indicative of the presence of two PhzS monomers in the asymmetric unit. A data set from a native protein crystal was collected to 2.4 Å

Table 1
Data-collection statistics.

Data were collected at beamlines ID14EH1 (native) and ID29 (Se-SAD) of the European Synchrotron Radiation Facility (ESRF, Grenoble, France). Values in parentheses are for the highest resolution shell.

	Native	Se-SAD
Wavelength (Å)	0.931	0.9791
Resolution (Å)	20.0–2.4 (2.5–2.4)	20.0–2.7 (2.8–2.7)
Space group	*C*2	*C*2
Unit-cell parameters (Å, °)	*a* = 144.2, *b* = 96.2, *c* = 71.7, α = γ = 90, β = 102.7	*a* = 70.6, *b* = 76.2, *c* = 80.2, α = γ = 90, β = 110.5
V_M (Å^3 Da^{-1})	2.8	2.3
Total measurements	141768 (15984)	40759 (4222)
Unique reflections	37329 (4260)	10933 (1129)
Average redundancy	3.8 (3.8)	3.8 (3.7)
$I/\sigma(I)$	18.6 (3.2)	11.3 (5.3)
Completeness (%)	99.7 (99.9)	98.9 (100)
Anomalous completeness† (%)	—	96.8 (97.1)
R_{sym}‡	5.7 (46.8)	7.5 (24.9)

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

resolution at 100 K in 110 non-overlapping 1° oscillation images at ID14EH1 of the European Synchrotron Radiation Facility (ESRF, Grenoble, France) using a MAR 165 CCD detector with 10 s exposure time. Data-collection statistics are given in Table 1.

SAD data from a seleno-L-methionine-labelled crystal were collected at 100 K from 360 non-overlapping 1° oscillations at λ = 0.975756 Å (the Se absorption edge) at ID29 of the same synchrotron using an ADSC Quantum (Q4R) detector with 1.5 s exposure time and three passes per image. These crystals also belong to space group *C*2; however, they possess different unit-cell parameters *a* = 70.6, *b* = 76.2, *c* = 80.2 Å, α = γ = 90, β = 110.5°, allowing the presence of only one PhzS monomer in the asymmetric unit. The crystals diffract to a resolution of 2.7 Å and are radiation-sensitive. Peaks in the Harker section at *v* = 0 indicate the presence of anomalous scatterers (Fig. 3).

All data were indexed, integrated and scaled with the *XDS* package (Kabsch, 1993). We are currently using these data to determine the position of Se atoms and derive initial phases for electron-density calculation.

Figure 3
Harker section at *v* = 0 contoured at 3σ, calculated from the anomalous differences of the Se-SAD data.

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