Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Preliminary crystallographic characterization of PrnB, the second enzyme in the pyrrolnitrin biosynthetic pathway

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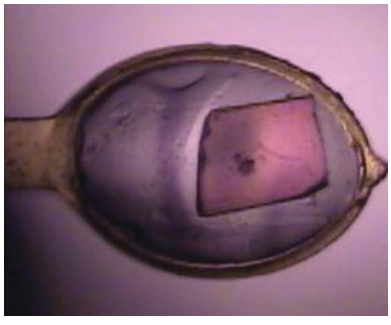
Received 18 September 2006
Accepted 9 October 2006

Pyrrolnitrin is the active ingredient of drugs for the treatment of superficial fungal infections and was used as a lead structure for the development of fludioxonil. It is an effective agent for plant diseases caused by the fungal pathogen *Rhizoctonia solani*. Pyrrolnitrin is made in four steps, the second of which, catalyzed by PrnB, is a novel chemical rearrangement of 7-chlorotryptophan. PrnB was overproduced in *Pseudomonas fluorescens* (BL915) and well diffracting crystals were obtained of a triple cysteine-to-serine mutant by sitting-drop vapour diffusion. Crystals grown in the presence of L-7-chlorotryptophan, D-tryptophan and L-tryptophan are reported. Data sets for each are reported with high-resolution limits of 2.0, 1.75 and 1.75 Å, respectively. Two crystals (PrnB in the presence of D-tryptophan and L-7-chlorotryptophan) belong to space group $C2$ with similar unit-cell parameters (a = 68.6, b = 79.5, c = 92.7 Å, $\alpha = \gamma = 90.0$, $\beta = 103.8^\circ$). Crystals grown in the presence of L-tryptophan belong to space group $C222_1$ and have unit-cell parameters a = 67.7, b = 80.1, c = 129.5 Å. All crystals contain a monomer in the asymmetric unit.

1. Introduction

Pyrrolnitrin [3-chloro-4-(2′-nitro-3′-chlorophenyl)pyrrole; Fig. 1] is a tryptophan-derived secondary metabolite with broad-spectrum antifungal activity which was first isolated in 1964 from *Pseudomonas pyrrocinia* (Arima *et al.*, 1964). Subsequently, pyrrolnitrin has been identified in several isolates of *Pseudomonas* and *Burkholderia* (previously named *Pseudomonas*). It has been implicated as an important mechanism of biological control of soil-borne fungal plant pathogens by these strains (Homma *et al.*, 1989; Howell & Stipanovic, 1979; Janisiewicz & Roitman, 1987). Pyrrolnitrin production has also been documented for strains of *Enterobacter agglomerans* (Chernin *et al.*, 1996), *Myxococcus fulvus* (Gerth *et al.*, 1982), *Corallococcus exiguous* (Gerth *et al.*, 1982), *Cystobacter ferrugineus* (Gerth *et al.*, 1982) and *Serratia* spp. (Kalbe *et al.*, 1996). Tryptophan was identified as a pyrrolnitrin precursor by feeding cultures with isotopically labelled compounds. Both D- and L-tryptophan isomers were efficiently incorporated into pyrrolnitrin. The pyrrolnitrin biosynthetic gene cluster from *P. fluorescens* (BL915) was finally identified in 1997 (van Pee & Ligon, 2000; Hammer *et al.*, 1997; Kirner *et al.*, 1998). This led to a proposal of the pathway for pyrrolnitrin biosynthesis (Fig. 1). The first and third steps are catalyzed by two related enzymes, PrnA and PrnC. Both PrnA and PrnC belong to the flavin-dependent halogenase superfamily. The structure and mechanism of PrnA have been elucidated (Dong *et al.*, 2005; Yeh *et al.*, 2006).

The transformation catalyzed by PrnB has no obvious chemical precedent. Although tryptophan-degrading enzymes such as indolamine dioxygenase and tryptophan dioxygenase are well known (Brady, 1975), both result in oxygen-containing compounds. This is not the case for PrnB; there is no additional oxygen in the product and PrnB decarboxylates its substrate. Sequence-comparison methods do not identify any protein with convincing homology to PrnB, further complicating any attempt to rationalize the chemical



mechanism. In order to probe the chemistry of the enzyme, we have initiated a crystallographic study.

2. Purification and site-directed mutagenesis

Genomic DNA was extracted from *P. fluorescens* (BL915) using the Qiagen Blood and Cell Culture DNA Kit. An overnight culture (10 ml) of *P. fluorescens* (BL915) was processed on a genomic DNA-purification column. Genomic DNA quality was assessed by running DNA gels. The DNA for PrnB was cloned using gene-specific primers and the clones were checked by sequencing. The verified PrnB cDNA was ligated into a variety of *Escherichia coli* expression vectors, but none gave soluble protein. The failure to express soluble PrnB in *E. coli* led us to investigate its expression in *P. fluorescens* (BL915). We employed the same *P. fluorescens* (BL915) Δ*ORF1-4* expression strain that had successfully been employed in the study of PrnA (Dong *et al.*, 2004, 2005; Keller *et al.*, 2000). In this strain, all four enzymes in the pyrrolnitrin pathway have been deleted from the chromosome. The *prnB* ORF was cloned into the pCIB-HIS vector, which was used for PrnA overexpression in *P. fluorescens* (BL915) Δ*ORF1-4* (Dong *et al.*, 2004, 2005; Keller *et al.*, 2000). This vector contains an N-terminal His tag. *E. coli* TOP10 cells were transformed with pCIB-HIS containing the PrnB insert and grown to produce a stock of high-quality plasmid DNA. Some plasmid was transformed into *E. coli* S17.1 strain. The *E. coli* strain was mixed with *P. fluorescens* (BL915) Δ*ORF1-4* and the plasmid was transferred between the bacteria by conjugation. Plasmid DNA was extracted from tetracycline-resistant clones of *P. fluorescens* (BL915) Δ*ORF1-4*. Clones carrying the correct plasmids were chosen for protein expression. *P. fluorescens* (BL915) Δ*ORF1-4* cells were grown as described previously (Dong *et al.*, 2004).

pCIB-HIS-*prnB* gave overexpression of soluble His-PrnB, which was confirmed by mass spectrometry. For analysis of *in vitro* activity, one part cells was resuspended in two parts 50 m*M* potassium phosphate buffer pH 7.2 and lysed by sonication. The addition of 100 µ*M* D/L-7-chlorotrytophan to the cell lysate containing His-PrnB led to the production of monodechloroaminopyrrolnitrin. The presence of this product was confirmed by HPLC analysis according to a previously published method (van Pee *et al.*, 1980; Hohaus *et al.*, 1997). Control samples (heat-treated or no His-PrnB) gave no such product. For purification of PrnB, cells were harvested by centrifugation and lysed by sonication on ice into 20 m*M* Tris–HCl pH 7.5, 100 m*M* NaCl. The soluble fraction was applied onto a 5 ml Ni^{2+} HiTrap column and eluted with an increasing gradient of imidazole (0–400 m*M*). Fractions containing His-PrnB were pooled before running on a Superdex S200 gel-filtration column. The protein ran inconsistently between batches, but always showed at least two peaks corresponding to a monomer and a dimer. The presence of 5 m*M* dithiothreitol (DTT) resulted in predominantly the monomeric species. This protein was concentrated to 16 mg ml^{-1}, but failed to give useful crystals using Nextal Classics, JSGS+ (Qiagen) and Hampton PEG/Ion crystallization kits.

The sensitivity of the gel-filtration profile to DTT suggested that PrnB was aggregating owing to disulfide-bond formation. As a result, we decided to mutate all three Cys residues to Ser (C21S, C60S and C175S). The *P. fluorescens* (BL915) PrnB triple mutant (*tm*PrnB) was created using the QuickChange Multi Site-Directed Mutagenesis Kit. Only one round of mutagenesis was required and the following primers were used (the mutation is marked in bold and the changed triplet is underlined): 5′-C21S, ACGCTGCCGTGGCGGCCT**C**C-

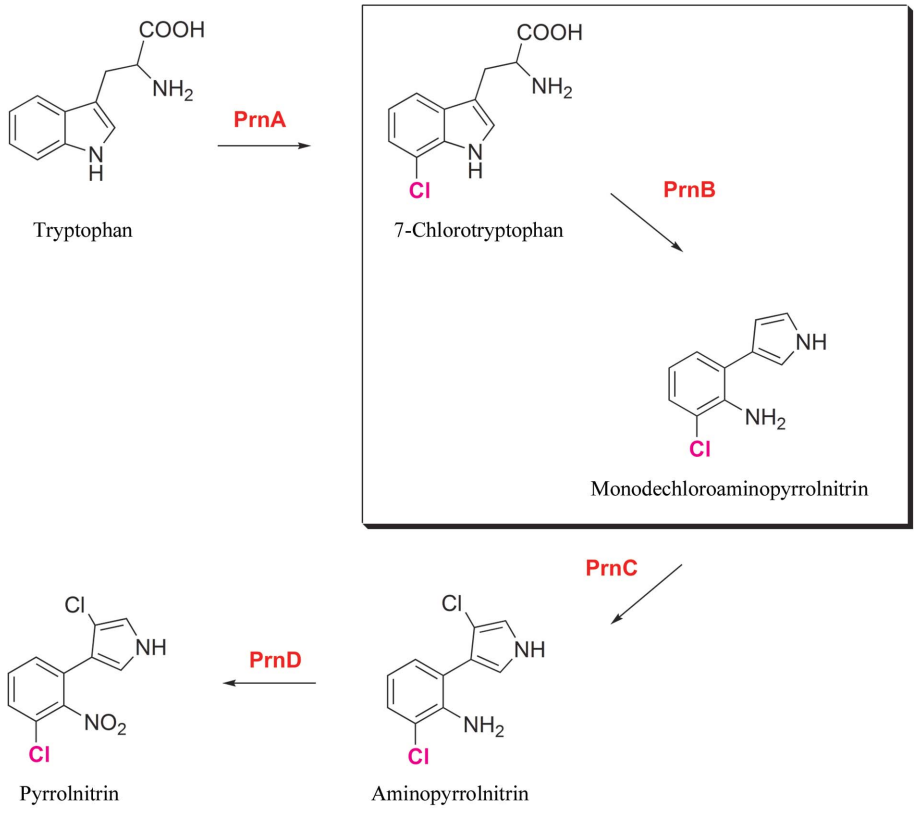


Figure 1
The biosynthetic pathway of pyrrolnitrin. The reaction catalyzed by PrnB is highlighted.

Table 1
X-ray data collection on PrnB.

Values in parentheses are for the outer shell.

	D-Trp	L-Trp	L-7-Cl-Trp
Resolution limits (Å)	51–1.75 (1.84–1.75)	38–1.75 (1.84–1.75)	51.0–1.90 (2.00–1.90)
Wavelength	0.9762	1.4880	1.2830
Unit-cell parameters (Å, °)	a = 68.2, b = 79.5, c = 92.3, $\alpha = \gamma = 90$, β = 103.4	a = 67.7, b = 80.1, c = 129.5, $\alpha = \beta = \gamma = 90$	a = 68.5, b = 79.5, c = 93.1, $\alpha = \gamma = 90$, β = 103.7
Space group	$C2$	$C222_1$	$C2$
Unique reflections	47277 (6791)	35216 (4879)	36544 (4608)
Multiplicity (%)	3.7 (3.8)	4.4 (4.0)	2.6 (1.8)
Completeness (%)	97.5 (96.3)	98.1 (94.3)	95.8 (83.0)
R_{merge}	0.073 (0.666)	0.082 (0.193)	0.065 (0.437)
$I/\sigma(I)$	12.7 (2.0)	16.0 (4.2)	13.0 (2.0)
Solvent content (%)			
With one monomer	55	39	55
With two monomers	12	<0	12

CGATCCGCTGCAGGC; 5′-C60S, GCGCGGCCTGCCCT**C**CGGCTGGGGTTTCGTCGAAGC; 5′-C175S. CGAGTTCGCGCAAAGGT**C**CGACGAGCTGGAAGCC; 3′-C21S, CGCGCCTGCAGCGGATCG**G**AGGCCGCCACGGCAGC; 3′-C60S, GACGAAACCCCAGCCG**G**AGGGCAGGCCGCGCACCGG; 3′-C175S, CTTCCAGCTCGTCG**G**ACCTTTGCGCGAACTCGG. *tm*PrnB was purified in the same way as native protein. It showed the same activity in feeding experiments. Crucially, gel filtration showed a single species consistent with a monomer independent of the addition of DTT.

3. Crystallization and X-ray data collection

For crystallization experiments, *tm*PrnB was concentrated to 16 mg ml^{-1} (measured by the Bradford assay) in 20 m*M* Tris–HCl pH 7.2. Saturated solutions of each enantiomer of tryptophan were used; saturated solutions were made by agitating excess solid material with the protein solution. The precise concentration of the tryptophan is therefore unknown. Crystallization trials were set up at the Scottish Structural Proteomic Facility with a nanodrop crystallization robot (Cartesian HoneyBee) on sitting-drop plates (Hampton Research CrystalQuick 96) using Nextal Classics, JCSG+ (Qiagen) and Hampton PEG/Ion crystallization kits. Each drop contained 200 nl protein solution plus 200 nl precipitant in vapour equilibrium with 100 µl precipitant. We failed to obtain crystals of protein unless *tm*PrnB was first incubated with tryptophan. Crystals were obtained from only one condition (during the four weeks of observation), which was the same for both D- and L-tryptophan. This crystallization condition was optimized independently for both incubations by increasing drop size and systematically varying the pH and precipitant concentration. The best results (judged by crystal appearance) were obtained using sitting-drop plates (Hampton Research CrystalClear Strips 96-well plates) with 1.5 µl protein solution (16 mg ml^{-1}) plus 1.5 µl precipitant [0.22 *M* magnesium sulfate, 16%(*w*/*v*) PEG 3350] irrespective of whether protein was incubated with D- or L-tryptophan. Crystals tended to grow (but not always) as cluster of plates/rods, both habits being visible within the same well. We have been able to grow crystals of protein incubated with L-7-chlorotryptophan (using the same method of saturating the solution) under the same conditions. Irrespective of the incubating ligand, the crystals typically measure 0.2 × 0.2 × <0.05 mm. A plate was detached using a loop from a crystal cluster obtained from protein pre-incubated with L-tryptophan. The plate was cryoprotected with 20% (*R*,*R*)-2,3-butanediol (Fig. 2). Data were collected at 100 K on Station 9.6 at the Synchrotron Radiation Source, UK (SRS) using an ADSC Quantum 4 CCD. A single plate-shaped crystal grown in the presence of D-tryptophan was cryoprotected in the same way and a data set was collected at 100 K on beamline ID29 using an ADSC Quantum 4 CCD. A single rod-shaped crystal grown in the presence of L-7-chlorotryptophan was cryoprotected in the same way and a data set was collected on Station 20 at the SRS using an ADSC Quantum 4 CCD. All three data sets were collected as a series of 0.5° oscillations. All images were indexed in *MOSFLM* (Leslie, 1992) and merged with *SCALA* (Evans, 1997) as implemented in the *CCP*4 package (Collaborative Computational Project, Number 4, 1994). Full statistics are given in Table 1. Interestingly, the unit cells of the L-tryptophan-incubated crystals appear to be related to the other two (with *a* and *b* common), but the data are distinct. The lower symmetry space group for the crystals grown in the presence of D-tryptophan and 7-chlorotryptophan appears to be correct; we failed to index either in higher symmetry. Matthews solvent-content calculations suggest that all crystals contain a monomer in the asymmetric unit, but the L-tryptophan-grown crystals contain significantly less solvent.

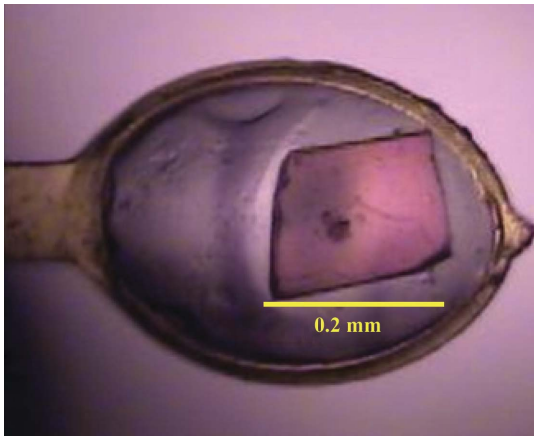


Figure 2
A crystal of PrnB grown in the presence of L-tryptophan and mounted for data collection.

4. Results and discussion

We have obtained good-quality crystals of PrnB from *P. fluorescens* (BL915) grown in the presence of substrate and two substrate mimics. The lack of homology of PrnB to proteins of known structure has ruled out molecular replacement as a possible method of structure solution. Therefore, we will have to use experimental phasing methods relying on the incorporation of anomalous scatters. We have shown the *P. fluorescens* (BL915) system can be used to incorporate selenium in much the same way as in *E. coli* (Dong *et al.*, 2004). As the crystals give high-quality data, we expect this to be straightforward. In both crystals, we have obtained the result that there is a monomer in the asymmetric unit. The mutation of Cys to Ser to prevent aggregation has been used in other systems (Pesce *et al.*, 2003; Vallone *et al.*, 2004) and was essential here. Once again, the *P. fluorescens* (BL915) expression system has provided a relatively simple method of rescuing apparently 'insoluble' protein from *E. coli* expression systems. The yield of the pure PrnB is around 5 mg from 1 l of culture. The system therefore possibly merits wider use in the structural biology community. We expect that a structural analysis of

PrnB will shed light on the chemical mechanism of this fascinating enzyme.

JHN is a BBSRC Career Development Fellow. The project was funded by a BBSRC grant on pyrrolnitrin biosynthesis to JHN.

References

Arima, K., Imanaka, H., Kousaka, M., Fukuda, A. & Tamura, G. (1964). *Agric. Biol. Chem.* **28**, 575–576.

Brady, F. O. (1975). *Bioinorg. Chem.* **5**, 167–182.

Chernin, L., Brandis, A., Ismailov, Z. & Chet, I. (1996). *Curr. Microbiol.* **32**, 208–212.

Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.

Dong, C. J., Flecks, S., Unversucht, S., Haupt, C., van Pee, K.-H. & Naismith, J. H. (2005). *Science*, **309**, 2216–2219.

Dong, C. J., Kotzsch, A., Dorward, M., van Pee, K.-H. & Naismith, J. H. (2004). *Acta Cryst.* D**60**, 1438–1440.

Evans, P. R. (1997). *Jnt CCP4/ESF–EACBM Newsl. Protein Crystallogr.* **33**, 22–24.

Gerth, K., Trowitzsch, W., Wray, V., Hofle, G., Irschik, H. & Reichenbach, H. (1982). *J. Antibiot.* **35**, 1101–1103.

Hammer, P. E., Hill, D. S., Lam, S. T., van Pee, K.-H. & Ligon, J. M. (1997). *Appl. Environ. Microbiol.* **63**, 2147–2154.

Hohaus, K., Altmann, A., Burd, W., Fischer, I., Hammer, P. E., Hill, D. S., Ligon, J. M. & van Pee, K.-H. (1997). *Angew. Chem. Int. Ed.* **36**, 2012–2013.

Homma, Y., Sato, Z., Hirayama, F., Konno, K., Shirahama, H. & Suzui, T. (1989). *Soil Biol. Biochem.* **21**, 723–728.

Howell, C. R. & Stipanovic, R. D. (1979). *Phytopathology*, **69**, 480–482.

Janisiewicz, W. J. & Roitman, J. (1987). *Phytopathology*, **77**, 1776–1776.

Kalbe, C., Marten, P. & Berg, G. (1996). *Microbiol. Res.* **151**, 433–439.

Keller, S., Wage, T., Hohaus, K., Holzer, M., Eichhorn, E. & van Pee, K.-H. (2000). *Angew. Chem. Int. Ed.* **39**, 2300–2302.

Kirner, S., Hammer, P. E., Hill, D. S., Altmann, A., Fischer, I., Weislo, L. J., Lanahan, M., van Pee, K.-H. & Ligon, J. M. (1998). *J. Bacteriol.* **180**, 1939–1943.

Leslie, A. G. W. (1992). *Jnt CCP4/ESF–EACBM Newsl. Protein Crystallogr.* **26**.

van Pee, K.-H. & Ligon, J. M. (2000). *Nat. Prod. Rep.* **17**, 157–164.

van Pee, K.-H., Salcher, O. & Lingens, F. (1980). *Angew. Chem. Int. Ed.* **19**, 828–829.

Pesce, A., Dewilde, S., Nardini, M., Moens, L., Ascenzi, P., Hankeln, T., Burmester, T. & Bolognesi, M. (2003). *Structure*, **11**, 1087–1095.

Vallone, B., Nienhaus, K., Brunori, M. & Nienhaus, G. U. (2004). *Proteins*, **56**, 85–92.

Yeh, E., Cole, L. J., Barr, E. W., Bollinger, J. M., Ballou, D. P. & Walsh, C. T. (2006). *Biochemistry*, **45**, 7904–7912.