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Crystallization, diffraction data collection and preliminary crystallographic analysis of DING protein from *Pseudomonas fluorescens*

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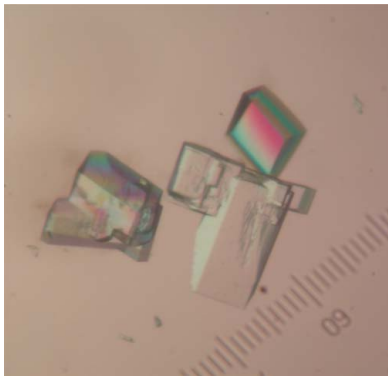

PfluDING is a phosphate-binding protein expressed in *Pseudomonas fluorescens*. This protein is clearly distinct from the bacterial ABC transporter soluble phosphate-binding protein PstS and is more homologous to eukaryotic DING proteins. Interestingly, bacterial DING proteins have only been detected in certain *Pseudomonas* species. Although DING proteins seem to be ubiquitous in eukaryotes, they are systematically absent from eukaryotic genomic databases and thus are still quite mysterious and poorly characterized. PfluDING displays mitogenic activity towards human cells and binds various ligands such as inorganic phosphate, pyrophosphate, nucleotide triphosphates and cotinine. Here, the crystallization of PfluDING is reported in a monoclinic space group ($P2_1$), with typical unit-cell parameters $a = 36.7$, $b = 123.7$, $c = 40.8$ Å, $\alpha = 90$, $\beta = 116.7$, $\gamma = 90^\circ$. Preliminary crystallographic analysis reveals good diffraction quality for these crystals and a 1.43 Å resolution data set has been collected.

1. Introduction

PstSs are 'soluble' phosphate-binding proteins of molecular weight ranging from 34 to 38 kDa that are part of the bacterial ABC cassette phosphate-uptake system. Crystallographic structures reveal that PstSs adopt a characteristic 'Venus flytrap' fold composed of two hinged domains around a conserved phosphate-binding site (Luecke & Quiocho, 1990; Vyas *et al.*, 2003).

Recently, eukaryotic proteins that show significant sequence homology between their N-terminal region and PstSs have been reported (Berna *et al.*, 2002). These proteins are named DING proteins owing to their conserved DINGGG N-terminal amino-acid sequence. Interestingly, DING proteins have been independently isolated from a wide range of eukaryotic sources: they have been identified in animals (Riah *et al.*, 2000; Weebadda *et al.*, 2001; Kumar *et al.*, 2004), plants (Berna *et al.*, 2002) and fungi (Chen *et al.*, 2007). For example, a human synovial DING protein was described by Hain *et al.* (1996) and a very similar protein has been characterized from human fibroblast and tumour cells (Adams *et al.*, 2002). A 38 kDa DING protein was isolated from breast carcinoma cells by genistein-agarose affinity chromatography (Belenky *et al.*, 2003). Human phosphate-binding protein (HPBP), the first DING protein to also be identified as a phosphate-binding protein, was purified from a human plasma lipoprotein fraction (Morales *et al.*, 2006). The first plant DING was identified in tobacco by virtue of its strong binding to an *Arabidopsis thaliana* germin-like protein (Berna *et al.*, 2002). Recently, a C-terminally shortened DING protein has been cloned and expressed from *Hypericum perforatum* (Darbinian-Sarkissian *et al.*, 2006). A DING protein has also been identified in a fungus (*Ganoderma lucidum*; Du *et al.*, 2007). Although DING proteins seem to be ubiquitous in eukaryotes, genes coding for DING proteins are systematically absent from genomic databases (Berna *et al.*, 2007). Of course, genes coding for this protein family are expected to exist and, although no complete nucleotide sequences are known, a few partial DNA sequences have been cloned or identified in un-annotated parts of genomes (Berna *et al.*, 2007). The only complete amino-acid sequence that is available is that of HPBP, which was completely sequenced using coupled crystallographic and mass-spectrometry analyses (H. Diemer, M. Elias, C. Schaeffer, F. Renault,

C. Contreras-Martel, A. Van Dorsselaer & E. Chabriere, in preparation). The crystallographic structure of HPBP complexed with a phosphate ion confirmed that the DING-protein family is related to PstS proteins and adopts the characteristic 'Venus flytrap' motif (Morales *et al.*, 2006).

Interestingly, some *Pseudomonas* species, but to our knowledge no other bacteria, have been shown to express DING proteins that are more homologous to eukaryotic DINGs (Berna *et al.*, 2007). Since these eukaryote-related DING proteins are expressed in particular secretory operons, they are clearly distinguishable from PstS. Furthermore, the low sequence identity (16.1%) between two members of these respective families (*P. fluorescens* DING and *Escherichia coli* Psts) confirms this fact. The eukaryote-related DING protein from *P. fluorescens* (PfluDING) was cloned and expressed. This 372-amino-acid protein was shown to bind phosphate and various ligands such as pyrophosphate, nucleotide triphosphates and cotinine with high affinity and to possess significant mitogenic activity towards human fibroblasts (Scott & Wu, 2005).

PfluDING is closely related to eukaryotic DINGs (71.3% sequence identity with HPBP) and displays very similar properties. Since the PfluDING gene is available, this protein is a good model for structure–function relationship studies on the DING-protein family.

In this paper, we report the crystallization of PfluDING in a monoclinic crystal form and preliminary crystallographic characterization.

2. Materials and methods

2.1. Protein expression and purification

Recombinant DING protein from *P. fluorescens* (PfluDING SBW25) was expressed in *E. coli* BL21 (DE3) as a fusion protein with a C-terminal hexahistidine tag and purified from bacterial lysates by affinity chromatography, as described previously (Scott & Wu, 2005). Based on SDS–PAGE experiments, the purity of the recombinant protein was estimated to be greater than 95%. Protein was dialyzed overnight against 20 m*M* Tris buffer pH 8.5 containing 5 m*M* β-mercaptoethanol and concentrated using a Centricon centrifugation system with a 3.5 kDa molecular weight cutoff membrane. Protein concentration was determined using UV spectrophotometry (ε_{280} = 42 650 M^{-1} cm^{-1}) and set to 10 mg ml^{-1} for crystallization experiments.

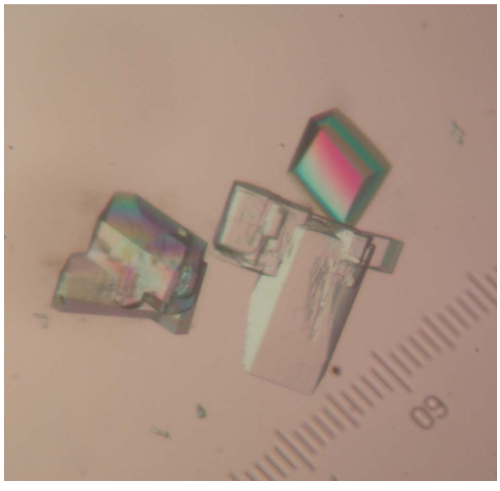

Figure 1
Typical PfluDING crystals. One division represents 20 µm.

2.2. Protein crystallization

Initial screening was performed at 293 K using sitting-drop vapour diffusion and the commercially available sparse-matrix screens (Jancarik & Kim, 1991) Crystals Screens I and II (Hampton Research Inc.) and Wizard Screens I and II (Emerald Biostructures). A micropipetting crystallization system (Mosquito, LabTech, UK) was used to test three protein:precipitant volume ratios (1:2, 2:2 and 2:1; one unit volume is 150 nl) for each crystallization condition. Condition No. 17 of Wizard Screen I [30%(*w*/*v*) PEG 8000, 100 m*M* acetate buffer pH 4.5 and 200 m*M* Li_2SO_4] turned out to be a promising crystallization condition and was then optimized using the hanging-drop vapour-diffusion method. Polynucleated crystals usually appear within a few days at high PEG concentrations (28–32%). After a week, PfluDING monocrystals had grown in 2 µl droplets of protein solution mixed with the same amount of reservoir solution [24–28%(*w*/*v*) PEG 8000, 100 m*M* acetate buffer pH 4.5 and 200 m*M* Li_2SO_4]. Monocrystals had typical dimensions of 0.10 × 0.10 × 0.05 µm (Fig. 1). Despite much effort, this protein has a tendency to form polynucleated crystals and seeding strategies are currently being tested to improve crystal quality.

2.3. Data collection and processing

Prior to data collection, crystals were soaked for a few seconds in a cryoprotective solution containing 10%(*w*/*v*) glycerol, 28% PEG 8000, 100 m*M* acetate buffer pH 4.5 and 200 m*M* Li_2SO_4. The crystal was then mounted on a loop (LithoLoop, Molecular Dimensions Ltd) and flash-cooled in liquid nitrogen. Data collection was performed at the BM14 UK MAD beamline (ESRF, Grenoble, France) using a wavelength of 0.9535 Å and a MAR CCD (Mosaic 225) detector. A complete data set was obtained by collecting 200 frames with an oscillation step of 1° and 20 s exposure time. Data were processed and scaled using the *XDS* package (Kabsch, 1993). Crystallographic analyses were performed using the *CCP*4 suite (Collaborative

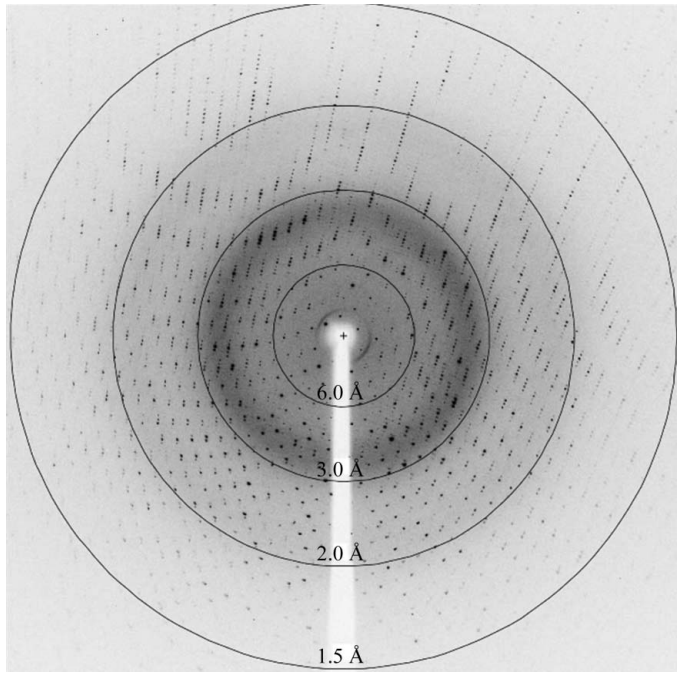

Figure 2
Typical diffraction pattern of a crystal of PfluDING. The edge of the frame is at 1.43 Å.

Table 1
Crystal data and intensity statistics.

Values in parentheses are for the last resolution shell.

Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 36.7, b = 123.7, c = 40.8, α = 90.0, β = 116.7, γ = 90.0
Temperature (K)	100
Wavelength (Å)	0.9535
Resolution range (Å)	60–1.43 (1.50–1.43)
No. of observed reflections	235730 (21073)
No. of unique reflections	58905 (7166)
Redundancy	4.0 (2.9)
Completeness (%)	98.1 (89.2)
R_{sym} (%)	2.6 (10.2)
Mean $I/\sigma(I)$	38.9 (10.52)

Computational Project, Number 4, 1994). Crystal data are given in Table 1.

3. Results

Despite its relatively moderate size, the PfluDING crystal exhibits remarkable diffraction quality, as indicated by the high resolution of the reported data set (1.43 Å; Fig. 2). Moreover, the very good data statistics at the high-resolution limit [R_{sym} of 10.2% and $I/\sigma(I)$ of 10.52 for the last shell] means that the diffraction potential of the crystal has not yet been fully exploited. The average thermal factor (B factor), a good indicator of diffraction quality, was estimated to be 8.9 Å^2 from the Wilson plot. Such a small value, in addition to previous observations, allows us to expect potential improvement in future data collections. Given the unit-cell parameters and the presence of 372 residues in the PfluDING molecule, the Matthews program indicated that the asymmetric unit contains only one PfluDING molecule. Thus, the calculated Matthews coefficient is 1.98 Å^3 Da^{-1}, which corresponds to 37.9% solvent content (Matthews, 1968). A consistent molecular-replacement solution has been found using the *MOLREP* program with HPBP (PDB code 2cap) as a model. The initial molecular-replacement solution (using data to 3.75 Å resolution) resulted in an R_{cryst} of 0.38 and a correlation value of 0.58. Refinement and interpretation of the structure of PfluDING at 1.43 Å resolution are currently in progress.

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