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Crystallization and preliminary X-ray crystallographic analysis of peptide deformylase (PDF) from *Bacillus cereus* in ligand-free and actinonin-bound forms

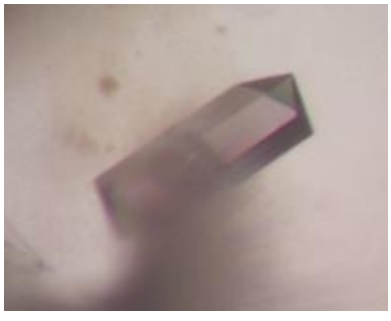
In bacteria, protein expression initiates with an *N*-formyl group and this needs to be removed in order to ensure proper bacterial growth. These formylation and deformylation processes are unique to eubacteria; therefore, inhibition of these would provide a novel antibacterial therapy. Deformylation is carried out by peptide deformylase (PDF). PDF from *Bacillus cereus*, one of the major pathogenic bacteria, was cloned into expression plasmid pET-28a (Novagen), overexpressed in *Escherichia coli* BL21 (DE3) and purified to high quality. Crystals have been obtained of both ligand-free PDF and PDF to which actinonin, a highly potent naturally occurring inhibitor, is bound. Both crystals belong to space group $P2_12_12_1$, with unit-cell parameters a = 42.72, b = 44.04, c = 85.19 Å and a = 41.31, b = 44.56, c = 84.47 Å, respectively. Diffraction data were collected to 1.7 Å resolution for the inhibitor-free crystals and to 2.0 Å resolution for the actinonin-bound crystals.

1. Introduction

In eubacteria, most mature proteins do not usually contain an N-terminal formyl group or methionine, although all nascent polypeptides are synthesized with *N*-formylmethionine at the N-terminus. The N-terminal methionine is removed by methionine aminopeptidase (EC 3.4.11.18; Adams, 1968; Pei, 2001, and references therein) after the formyl moiety has been removed by a metallohydrolase called peptide deformyalse (PDF; EC 3.5.1.31). The distribution of PDF-gene homologues has been studied and it has been concluded that the gene encoding PDF (*def*) is present in all sequenced pathogenic bacterial genomes; this defomylation step has been shown to be essential for bacterial growth in *Escherichia coli*, *Streptococcus pneumoniae* and *Staphylococcus aureus* (Mazel *et al.*, 1994; Meinnel & Blanquet, 1994; Margolis *et al.*, 2000; Apfel *et al.*, 2001). Several eukaryotic parasites such as *Plasmodium falciparum* and plants also have a PDF-gene homologue; however, PDF activity in humans is considered to be an evolutional remnant without function (Giglione *et al.*, 2000; Serero *et al.*, 2001; Nguyen *et al.*, 2003).

The aforementioned properties suggest that PDF could provide the basis for discovering broad-spectrum antibacterial drugs and this has attracted a number of pharmaceutical companies and groups to develop inhibitors targeting PDF. So far, several classes of inhibitors have been reported (Yuan *et al.*, 2001; White *et al.*, 2003 and references therein). The crystal structures of PDFs from both Gram-positive and Gram-negative pathogens as well as the structures of PDF complexed with inhibitors have also been reported, thus providing a structural platform for the structure-based drug design of antibacterial agents (Groche *et al.*, 1998; Guilloteau *et al.*, 2002; Kreusch *et al.*, 2003; Smith *et al.*, 2003; Robien *et al.*, 2004; Zhou *et al.*, 2004, and references therein). Although PDF enzymes exhibit rather a low level of sequence similarity, they are classified into two types based on systematic search and analysis of bacterial genomes (Guilloteau *et al.*, 2002).

Bacillus cereus is an aerobic endospore-forming Gram-positive bacterium found in soil, on vegetables and in many raw and processed foods. The genome of *B. cereus* ATCC 14579 has been reported recently (Ivanova *et al.*, 2003) and is similar to *B. anthracis*, the causative agent of anthrax, which was implicated in bioterror attacks in the USA (Read *et al.*, 2003). Consumption of foods that contain



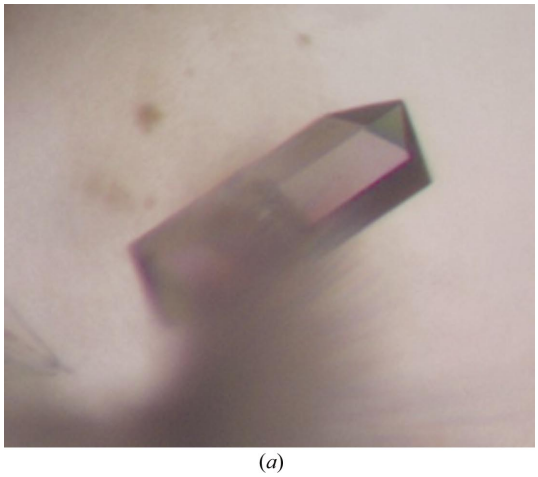
greater than 10^6 *B. cereus* per gram may result in food poisoning, typically abdominal pain and diarrhoea as well as an acute attack of nausea and vomiting a few hours to a day after consumption of contaminated food (Rhodelhamel & Harmon, 1998). *B. cereus* possesses two functional *def*-like gene products as in some other Gram-positive bacteria. For example, *B. subtilis* contains two *def*-like genes (*def* and *ykrB*) and YkrB is reported to be the main PDF in *B. subtilis* (Haas *et al.*, 2001).

Actinonin is a naturally occurring antibiotic that shows inhibitory activity against bacterial growth through the inhibition of PDF activity. We have crystallized *B. cereus* PDF as well as co-crystallized the enzyme in complex with actinonin.

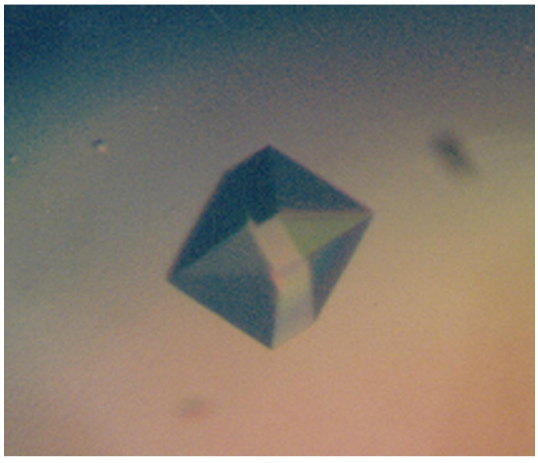
2. Experimental methods

2.1. Construction and expression

The *B. cereus def* gene encodes a 156-residue (MW = 17 480 Da) protein. The PDF gene from *B. cereus* ATCC 14579 was amplified by the polymerase chain reaction (PCR) using the genomic DNA as template. The designed forward and reverse oligonucleotide primers were 5′-GCC GTG ACG CAT ATG GCA GTT TTA GAA ATA ATA AAG CAT-3′ and 5′-GCT AGG CAT CTC GAG TCA CTA TTC TAG TTC GTT TTC TTC-3′. The designed primers have sequences for *Nde*I and *Xho*I restriction-endonuclease sites. For amplification, 1 µg of genomic DNA template was used and 0.2 m*M* dNTP (TaKaRa, Japan), 1 unit of Ex Taq polymerase (TaKaRa, Japan) and 0.5 µ*M* primer were added. 30 reaction cycles of 30 s at 367 K, 30 s at 329 K and 60 s at 345 K were carried out, followed by an additional 7 min incubation at 345 K in T-gradient thermo-blocks (Biometra, Germany). The PCR product was purified using the Bio101 gene clean kit (Bio101, USA). The PCR product was digested using *Nde*I and *Xho*I enzymes and was then inserted into the *Nde*I/*Xho*I-digested expression vector pET-28a. This construction added a hexahistidine tag at the N-terminus of the recombinant PDF.



(*a*)



(*b*)

Figure 1
(*a*) A crystal of *B. cereus* peptide deformylase. Its approximate dimensions are 0.2 × 0.2 × 0.6 mm. (*b*) A crystal of *B. cereus* peptide deformylase grown in the presence of actinonin, with approximate dimensions 0.4 × 0.2 × 0.4 mm.

The protein was overexpressed in *E. coli* BL21 (DE3) cells. The cells were grown in Luria–Bertani (LB) medium to an OD_{600} of 0.5 at 310 K and expression of the recombinant enzyme was induced by the addition of 0.5 m*M* isopropyl β-D-thiogalactopyranoside (IPTG) and 0.1 m*M* $NiSO_4$ at 291 K. Cell growth continued at 291 K for 16 h after IPTG induction and cells were harvested by centrifugation at 4200*g* (6000 rev min^{-1}; Sorvall GSA rotor) for 30 min at 277 K. The cell pellet was resuspended in ice-cold lysis buffer (50 m*M* Tris–HCl pH 8.0, 300 m*M* NaCl and 2.5% glycerol) and homogenized by sonification. The crude lysate was centrifuged for 30 min at 277 K and the supernatant was passed through a 0.45 µm filter to remove cell debris.

2.2. Purification and actinonin complex formation

The purification first utilized the N-terminal histidine tag using an Ni^+-chelated Hi-trap chelating column (Amersham Biosciences). The next step was treatment with thrombin in order to remove the N-terminal hexahistidine tag. The final purification step was gel-filtration on a Superdex-75 prep-grade column (Amersham Biosciences), which was previously equilibrated with a buffer containing 50 m*M* Tris–HCl pH 7.5, 150 m*M* NaCl and 1 m*M* DTT. The purified protein solution was concentrated to 10.0 mg ml^{-1} using an Amicon Ultra-15 (Millipore). The protein concentration was estimated by measuring the absorbance at 280 nm, employing the calculated extinction coefficient 5120 M^{-1} cm^{-1} (SWISS-PROT; http://www.expasy.ch/).

PDF protein in 50 m*M* Tris–HCl pH 7.5, 150 m*M* NaCl and 1 m*M* DTT was mixed with 2.5 m*M* actionin and incubated on ice for 1 h. The actinonin stock solution was prepared at 100 m*M* in 100% DMSO. The complex solution was centrifuged at 9800*g* for 30 min prior to the crystallization setup.

2.3. Crystallization

Initial screening for crystallization was performed on ligand-free PDF by the sitting-drop vapour-diffusion method using 96-well Intelli plates (Hampton Research) and a Hydra II Plus One (Matrix Technology) at 295 K. The protein concentration was 10.0 mg ml^{-1} in 50 m*M* Tris–HCl pH 7.5, 150 m*M* NaCl and 1 m*M* DTT. A sitting drop was prepared by mixing 200 nl each of the protein solution and the reservoir solution was equilibrated against 70 µl reservoir solution. The initial search for crystallization conditions was performed using commercially available kits from Hampton Research, Jena Bioscience and Emerald Biostructures. Out of the 1200 conditions screened, several microcrystals were obtained after 3–5 d: thin needle clusters from Hampton Grid Screen PEG 6000 condition No. 3 (5% PEG 6000, 0.1 *M* MES pH 6.0) and Hampton Grid Screen MPD condition No. 3 (10% MPD, 0.1 *M* MES pH 6.0) and JBS Screen 1 No. 2 (15% PEG 400, 0.1 *M* Na MES pH 6.5).

These conditions were optimized using hanging-drop vapour-diffusion experiments. Each hanging drop was prepared by mixing 2 µl each of the protein solution and the reservoir solution and was

Table 1
Data-collection statistics.

Values in parentheses are for the outermost resolution shell (1.76–1.70 Å for native PDF and 2.07–2.00 Å for the actinonin complex).

	PDF	Actinonin–PDF complex
X-ray wavelength (Å)	1.54178 (FR591 rotating anode)	1.12174 (Pohang, BL-6B)
Resolution range (Å)	30.0–1.70	50.0–2.00
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters (Å)	a = 42.719, b = 44.040, c = 85.185	a = 41.310, b = 44.558, c = 84.474
Total/unique reflections	74783/17740	47012/10299
Completeness (%)	96.9 (96.9)	92.3 (58.2)
Mean $I/\sigma(I)$ (%)	4.7 (1.0)	25.2 (4.1)
$R_{\rm merge}$† (%)	7.2 (38.4)	6.5 (19.1)

† $R_{\rm merge} = \sum_h \sum_i |I(h,i) - \langle I(h)\rangle| / \sum_h \sum_i I(h,i)$, where $I(h,i)$ is the intensity of the ith measurement of reflection h and $\langle I(h)\rangle$ is the mean value of $I(h,i)$ for all i measurements.

equilibrated over 0.5 ml reservoir solution. Diffraction-quality PDF crystals were obtained with a reservoir solution consisting of 2% PEG 6000, 0.1 *M* MES pH 5.5.

For crystallization of the actinonin–PDF complex, the hanging-drop vapour-diffusion method was used and conditions similar to those used in the ligand-free PDF optimization were screened. Each drop was prepared by mixing 1 µl each of the protein–inhibitor complex and reservoir solutions, and was equilibrated over 0.5 ml reservoir solution. Single crystals were obtained after a week from 6–10% PEG 400, 0.1 *M* MES pH 5.2–5.4.

2.4. Data collection

A native PDF crystal measuring 0.2 × 0.2 × 0.6 mm from 2% PEG 6000, 0.1 *M* MES pH 5.5 (Fig. 1) grown in 7 d was used for data collection. The crystal mounted in a cryoloop was dipped in cryoprotectant solution [2% PEG 6000, 0.1 *M* MES pH 5.5 and 25%(*v*/*v*) glycerol] for 2 s and placed immediately in a 100 K nitrogen-gas stream. X-ray diffraction data were collected using an R-AXIS IV^{++} image plate (Rigaku Corporation, Tokyo, Japan) mounted on a FR591 rotating-anode generator operated at 50 kV and 100 mA with Confocal Maxflux Optics (MSC) and X-Tream cryosystem (Rigaku Corporation, Tokyo, Japan). The crystal was rotated through a total of 120°, with 1.0° oscillation per frame. The program *CrystalClear* v.1.3 was used for processing and scaling. Attempts were made to collect data using synchrotron radiation, but the resolution did not improve.

The diffraction data from a PDF–actinonin complex crystal measuring 0.4 × 0.2 × 0.4 mm were collected at 100 K with a Bruker AXS Proteum300 CCD detector (Madison, WI, USA) at beamline 6B of Pohang Light Source. The wavelength of the synchrotron X-rays was 1.12174 Å. The crystal was rotated through a total of 150°, with 1.0° oscillation per frame. The raw data were processed and scaled using the program *HKL*2000 (Otwinowski & Minor, 1997).

3. Results

The recombinant *B. cereus* PDF with an N-terminal hexahistidine tag was expressed in *E. coli* BL21 (DE3) as a soluble fraction, with a yield of about 3 mg of highly purified protein per litre of culture. Native PDF crystals of approximate dimensions of 0.2 × 0.2 × 0.6 mm were obtained within 7 d using a reservoir solution consisting of 2% PEG 6000, 0.1 *M* MES pH 5.5, while the actinonin-complex crystals were obtained from 6–10% PEG 400, 0.1 *M* MES pH 5.2–5.4 in 7–10 d.

The native crystal of the ligand-free PDF belongs to space group $P2_12_12_1$, with unit-cell parameters a = 42.71, b = 44.04, c = 85.19 Å. The native data set is 96.9% complete to 1.7 Å resolution. The presence of one subunit of PDF in the asymmetric unit gives a crystal volume per protein weight ($V_{\rm M}$) of 2.29 Å^3 Da^{-1}, with the corresponding solvent content of 46.3% (Matthews, 1968).

The PDF–actinonin crystal also belongs to space group $P2_12_12_1$, with similar unit-cell parameters a = 41.31, b = 44.56, c = 84.47 Å. The data set from the complex is 92.3% complete to 2.0 Å resolution. The asymmetric unit contains one PDF–inhibitor molecule and the specific volume ($V_{\rm M}$) is 2.22 Å^3 Da^{-1}, with a solvent content of 44.6%.

Statistics for data collection are summarized in Table 1. The structure solved by molecular replacement using PDF from *Thermotoga maritime* (PDB code 1lme; Kreusch *et al.*, 2003) as a search model reveals clear electron density at the active sites and the details will be described elsewhere.

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References

Adams, J. M. (1968). *J. Mol. Biol.* **33**, 571–589.
Apfel, C. M., Locher, H., Evers, S., Takács, B., Hubschwerlen, C., Pirson, W., Page, M. G. P. & Keck, W. (2001). *Antimicrob. Agents Chemother.* **45**, 1058–1064.
Giglione, C., Serero, A., Pierre, M., Biosson, B. & Meinnel, T. (2000). *EMBO J.* **19**, 5916–5929.
Groche, D., Becker, A., Schlichting, I., Kabsch, W., Schultz, S. & Wagner, A. F. V. (1998). *Biochem. Biophys. Res. Commun.* **246**, 342–346.
Guilloteau, J.-P., Mathieu, M., Giglione, C., Blanc, V., Dupuy, A., Chevrier, M., Gil, P., Famechon, A., Meinnel, T. & Mikol, V. (2002). *J. Mol. Biol.* **320**, 951–962.
Haas, M., Beyer, D., Gahlmann, R. & Freiberg, C. (2001). *Microbiology*, **147**, 1783–1791.
Ivanova, N. *et al.* (2003). *Nature (London)*, **423**, 87–91.
Kreusch, A., Spraggon, G., Lee, C. C., Klock, H., McMullan, D., Ng, K., Shin, T., Vincent, J., Warner, I., Ericson, C. & Lesley, S. A. (2003). *J. Mol. Biol.* **330**, 309–321.
Margolis, P. S., Hackbarth, C. J., Young, D. C., Wang, W., Chen, D., Yuan, Z., White, R. & Trias, J. (2000). *Antimicrob. Agents Chemother.* **44**, 1825–1831.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Mazel, D., Pochet, S. & Marliere, P. (1994). *EMBO J.* **13**, 914–923.
Meinnel, T. & Blanquet, S. (1994). *J. Bacteriol.* **176**, 7387–7390.
Nguyen, K. T., Hu, X., Colton, C., Chakrabarti, R., Zhu, M. X. & Pei, D. (2003). *Biochemistry*, **42**, 9952–9958.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–332.
Pei, D. (2001). *Emerg. Ther. Targets*, **5**, 23–40.
Read, T. D. *et al.* (2003). *Nature (London)*, **423**, 81–86.
Rhodelhamel, E. J. & Harmon, S. M. (1998). In *Food and Drug Administration Bacteriological Analytical Manual*, 8th ed. (revision A), edited by R. L. Merker. Gaithersburg, MD, USA: AOAC International.
Robien, M. A., Nguyen, K. T., Kumar, A. V., Hirsh, I., Turley, S., Pei, D. & Hol, W. G. J. (2004). *Protein Sci.* **13**, 1155–1163.
Serero, A., Giglione, C. & Meinnel, T. (2001). *J. Mol. Biol.* **314**, 695–708.
Smith, K. J., Petit, C. M., Aubart, K., Smyth, M., McManus, E., Jones, J., Fosberry, A., Lewis, C., Lonetto, M. & Christensen, S. B. (2003). *Protein Sci.* **12**, 349–360.
White, R. J., Margolis, P. S., Trias, J. & Yuan, Z. (2003). *Curr. Opin. Pharmacol.* **3**, 502–507.
Yuan, Z.,Trias, J. & White, R. J. (2001). *Drug Discov. Today*, **6**, 954–961.
Zhou, Z., Song, X., Li, Y. & Gong, W. (2004). *J. Mol. Biol.* **339**, 207–215.