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Cloning, high-level expression, purification and crystallization of peptide deformylase from *Leptospira interrogans*

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A new peptide deformylase (PDF; EC 3.5.1.27) gene from *Leptospira interrogans* was identified and cloned into expression plasmid pET22b(+) and was highly expressed in *Escherichia coli* BL21(DE3). With DEAE-Sepharose anion-exchange chromatography followed by Superdex G-75 size-exclusion chromatography, 60 mg of PDF from *L. interrogans* was purified from 1 l of cell culture. Crystallization screening of the purified enzyme resulted in two crystal forms, from one of which a 3 Å resolution X-ray diffraction data set has been collected.

1. Introduction

In prokaryotes, the translation apparatus appears to be simpler than that in eukaryotes. However, in prokaryotes translation initiation involves an additional step of formylation of methionyl-$tRNA_f^{Met}$ and the occurrence of this modification of the first incorporated methionine is conserved throughout evolution (Adams & Capecchi, 1966; Meinnel & Blanquet, 1993). The enzyme responsible for the removal of the *N*-formyl moiety from nascent protein is peptide deformylase (PDF; Adams, 1968). The majority of polypeptides display other amino acids at their N-termini, which indicates that the *N*-formyl group is removed post- or co-translationally (Bradshaw *et al.*, 1998; Giglione, Pierre *et al.*, 2000). It is believed that the enzyme does not occur in eukaryotic cells and its pivotal role in bacterial cells makes it an attractive target for the development of new antibiotics (Meinnel, 2000).

PDFs are built around three well conserved motifs. The two most important motifs (EGCLS and HE*X*DH*XX*G) contain three metal ligands. In addition to the two above sequences, a third, G*X*G*X*AA*X*Q, is also strictly conserved (Meinnel *et al.*, 1997). Much recent research on PDF inhibitors centred on the substrate specificity of this enzyme and its catalytic mechanism have been reported (Wei & Pei, 2000; Jayasekera *et al.*, 2000; Durand *et al.*, 1999; Green *et al.*, 2000). The three-dimensional structures of *E. coli* PDF and its inhibitor complex have been determined by X-ray crystallography (Chan *et al.*, 1997; Hao *et al.*, 1999; Becker, Schlichting, Kabsch, Groche *et al.*, 1998; Becker, Schlichting, Kabsch, Schultz *et al.*, 1998; Clements *et al.*, 2001). To date, all structural and functional studies have been focused on *E. coli* PDF; no detailed structures are available for PDFs from pathogenic bacteria.

A ubiquitous environmental bacterium, *Leptospira*, causes an acute febrile illness occurring in humans or animals in all parts of the world. Here, we report the identification of a PDF gene from *L. interrogans* and the high-level expression, purification and crystallization of PDF from *L. interrogans* (LiPDF).

2. Experiment methods

2.1. Construction and expression

The complete *L. interrogans* (serovar *icterohaemorrhagiae*, strain Lai) genome was provided by the Chinese National Human Genome Center at Shanghai (CHGC). An efficient overexpression vector of the PDF gene was constructed by amplification of the *L. interrogans* genome with the help of two oligonucleotides. Lep1, 5′-GGAATTCCATATGTCAGTCAGAAAAAT-3′, created an *Nde*I restriction site at the level of the initiation codon. Lep2, 5′-GCGCTCGAGTTAGTCTAGGACGTTG-3′, introduced an *Xho*I restriction site downstream of the termination codon. The amplified fragment was cloned into a pET22b(+) vector (Novagen) and was expressed in host strain *E. coli* BL21(DE3). Recombinant DNA techniques were performed essentially as described by Sambrook *et al.* (1989). Expression was performed in 1 l LB medium (100 µ*M* $ZnCl_2$ was added), which was incubated at 310 K until OD_{600} reached about 0.6. The cultures were induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 800 µ*M* and incubation at 310 K for an additional 4 h. For preparation of soluble protein fractions, the cells from 1 l of

culture were first resuspended in 20 ml of cold (277 K) buffer *A* (50 m*M* HEPES pH 7.5, 10 m*M* NaCl, 100 µg ml^{-1} phenylmethanesulfonyl fluoride, 100 µ*M* $ZnCl_2$) and then lysed by sonication on ice. The clear supernatant with the soluble proteins was collected by centrifugation. All following purification steps were performed at 277 K.

2.2. Purification

An AKTA FPLC system was used (Amersham-Pharmacia). A 2.5 × 16 cm column of DEAE-Sepharose was eluted with buffer *B* (50 m*M* Tris–HCl pH 8.0, 10 m*M* NaCl, 3 m*M* DTT, 100 µ*M* $ZnCl_2$) plus a linear gradient of 10–500 m*M* NaCl. Fractions with PDF activity were concentrated and applied to a 2.6 × 60 cm column containing Superdex G-75 pre-equilibrated with buffer *C* (50 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl, 2 m*M* DTT, 100 µ*M* $ZnCl_2$). Fractions containing PDF were analyzed by SDS–PAGE and pooled for subsequent steps. Final protein concentration was determined by the Bio-Rad protein assay with BSA as the standard. Measurement of zinc by atomic absorption spectroscopy was carried out by flame atomic absorption spectrometry (Perkin–Elmer).

2.3. PDF activity assay

The standard PDF assay was coupled to formate dehydrogenase (FDH) essentially as described previously (Ragusa *et al.*, 1998). PDF activity was assayed by measuring the increase in absorbance of NADH at 345 nm as a function of time. The standard reaction mixture (final volume 200 µl) contained 50 m*M* HEPES pH 7.5, 0.1–1.5 m*M* For-Met-Ala-Ser (Balchem), 12 m*M* NAD^+ (Boehringer), 1 unit of yeast FDH (Sigma) and 40 µg bovine serum albumin (Fraction V, S_{abc}) which prevented any unspecific absorption to dilution tubes. PDF was diluted as necessary. Reaction was started by the addition of 2 µl of the diluted PDF to the reaction mixture and the OD_{345} was immediately monitored with an UV–VIS spectrophotometer. The kinetic parameters (K_m and k_{cat}) were derived from iterative non-linear least fits of the Michaelis–Menten equation using the experimental data.

2.4. Crystallization and data collection

Crystallization was carried out with the hanging-drop vapour-diffusion method using a sparse-matrix screen of 50 conditions from Hampton Crystal Screen I (Jancarik & Kim, 1991). Droplets containing 1 µl of protein solution (15 mg ml^{-1} protein in 50 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl, 2 m*M* DTT and 100 µ*M* $ZnCl_2$) were mixed with 1 µl of well solution. Initial crystallization trials were carried out at two different temperatures (277 and 291 K), but the protein precipitated in nearly all of the drops overnight at 291 K. Further crystal-growth experiments were therefore performed at 277 K. Condition 33 in Crystal Screen I, which contained 4 *M* sodium formate, produced good LiPDF crystals (form *A*); condition 31, which contained 18%(*w*/*v*) PEG 4000 and 0.2 *M* $(NH_4)_2SO_4$, produced small LiPDF crystals (form *B*). Form *A* crystals grew to a maximum size of 0.8 × 0.8 × 0.3 mm in a week. By varying the PEG 4000 concentration, form *B* crystals with a maximum size of 0.6 × 0.4 × 0.3 mm were obtained with well solution consisting of 12%(*w*/*v*) PEG 4000 and 0.2 *M* $(NH_4)_2SO_4$. X-ray diffraction data were collected from crystal form *A* on a MAR Research image-plate system with a local X-ray source of 2.0 kW at room temperature. The wavelength was 1.5418 Å and the exposure time was 15 min per image. The oscillation step for each image was 1°. The crystal-to-detector distance was set to 220 mm. The data were processed with *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). Self-rotation peaks were searched with *X-PLOR* (Brünger, 1992).

Table 1
Statistics of data collection and processing of crystal form *A*.

Space group	$P4_12_12$ or $P4_32_12$
Unit-cell parameters (Å)	$a = b = 83.9$, $c = 204.4$
Resolution (Å)	3.0
No. of reflections	58810
No. of independent reflections	13456
Completeness (last shell) (%)	87.7 (85.2)
R_{merge} (last shell)	0.109 (0.384)

3. Results

3.1. Sequence comparison, purification and activity assay

The PDF gene from *L. interrogans* was verified by DNA-sequence analysis. The sequence has been deposited in GenBank (GenBank Accession Number AY040678). PDF sequences from *E. coli* and *L. interrogans* are aligned in Fig. 1.

Each step of the purification procedure, as monitored by SDS–PAGE, is shown in Fig. 2. Recombinant protein was obtained with a final yield of approximately 60 mg pure LiPDF from 1 l of cell culture in the two-step chromatographic procedure. The purified LiPDF showed significant deformylase activity. The K_m value of LiPDF towards For-Met-Ala-Ser was 2.4 m*M* and k_{cat} was about 5 s^{-1}. The high yield and simple purification procedure make it suitable for further crystallographic studies.

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                                                          Motif 1
E.coli PDF   MSVLQVLHIPDERLRKVAKPVEE---VNAEIQRIVDDMFETMYAEEGIGLAATQVDIHQR   57
LiPDF        MSVRKILRMGDPILRKISEPVTEDEIQTKEFKKLIRDMFDTMRHAEGVGLAAPQIGILKQ   60
             ***   *   *  ***   ** *      *      *** **   ** **** *  *

                                                  Motif 2
E.coli PDF   IIVIDVSENR--------DERLVLINPELLEKSGET-GIEEGCLSIPEQRALVPRAEKVK   108
LiPDF        IVVVGSEDNERYPGTPDVPERIIL-NPVITPLTKDTSGFWEGCLSVPGMRGYVERPNQIR   119
             * *     *          **    **          *  ***** *  *  * *

                                        Motif 3
E.coli PDF   IRALDRDGKPFELEADGLLAICIQHEMDHLVGKLFMDYLSPLKQQRIRQKVEKLDRLKARA 169
LiPDF        MQWMDEKGNQFDETIDGYKAIVYQHECDHLQGILYVDRLKDTKLFGFNETLDSSHNVLD   178
                 *  *  *    **  **  *** *** * *  * *
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Figure 1
Sequence alignment of *E. coli* PDF and LiPDF. The conserved residues are marked with asterisks. The three previously defined motifs are underlined and labelled.

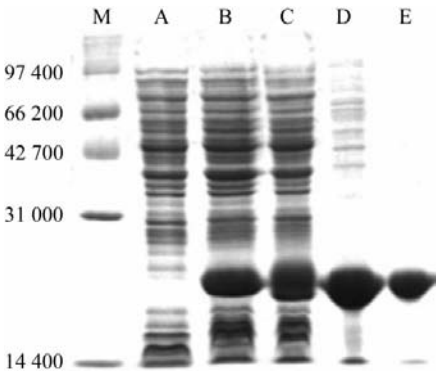

Figure 2
SDS–PAGE electrophoresis of LiPDF during purification. Proteins were analyzed on 15% SDS–PAGE stained with Coomassie blue. M, marker; Lane A, crude cell lysates before IPTG induction; lane B, crude cell lysates after 0.8 m*M* IPTG induction – PDF is expressed highly; lane C, clear supernatant; lane D, purified PDF after DEAE-Sepharose chromatography; lane E, purified PDF after Superdex G-75 chromatography

3.2. Crystallization and preliminary X-ray analysis

A complete diffraction data set to 3 Å resolution has been collected from a crystal of form *A*. The systematic absences showed that the crystal belonged to space group $P4_12_12$ or $P4_32_12$, with unit-cell parameters $a = b = 83.9$, $c = 204.4$ Å (Table 1). The V_M value is 2.2 Å^3 Da^{-1}, assuming four LiPDF molecules per asymmetric unit. Calculation of the self-rotation function using *X-PLOR* showed a strong non-crystallographic twofold symmetry with $\psi = 48.7$, $\varphi = 28.6$, $\kappa = 179.2^\circ$. However, another non-crystallographic twofold axis was not clear. Form *B* crystals diffracted to 4 Å resolution but with very high mosaicity, implying the crystal packing was not well ordered.

4. Discussion

LiPDF reported here was shown to bind a zinc ion at a ratio of 1:1 by atomic absorption measurement. Its K_m value is as low as that of Ni^{2+}-PDF from *E. coli* (K_m = 3.3–4.5 m*M*) and much lower than that of Zn^{2+}-PDF from *E. coli* (K_m = 50–90 m*M*). According to the k_{cat}/K_m ratio, Zn^{2+}-LiPDF has a much higher catalytic efficiency than *E. coli* Zn^{2+}-PDF. The structural basis for the differences in substrate affinity is of interest and would provide deeper insight into the mechanism of PDF and would help in the design of more specific inhibitors. It has been reported that PEG may bind in the PDF catalytic pocket (Becker, Schlichting, Kabsch, Groche *et al.,* 1998). The form *B* crystals obtained under conditions including PEG 4000 (and other PEGs of different molecular weight) could help in understanding the substrate-binding properties of LiPDF. The improvement of form *B* crystals is also in progress.

Recent bioinformatic analysis of the data produced by the systematic sequencing of genomes has provided new views of PDF expression in the eukaryotic kingdom. More surprising was their discovery in the expressed sequence tag (EST) in the mouse and human genomes (Giglione, Serero *et al.*, 2000). Little is known about the roles of PDF in eukaryotes. Thus, further investigations focusing on PDFs from different organisms are needed.

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