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Co-crystallization of *Leptospira interrogans* peptide deformylase with a potent inhibitor and molecular-replacement schemes with eight subunits in an asymmetric unit

Zhaocai Zhou[a,b] and Weimin Gong[a,b]*

[a]School of Life Sciences, Key Laboratory of Structural Biology, University of Science and Technology of China, Hefei, Anhui 230026, People's Republic of China, and [b]National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, People's Republic of China

Correspondence e-mail: wgong@sun5.ibp.ac.cn

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Translation initiation in eubacteria involves a formylmethionine at the N-terminus of newly synthesized polypeptides. This N-formyl group is removed by peptide deformylase (PDF) during the post-translation process. Such a formylation/deformylation cycle is essential for the cell survival of eubacteria, but is not utilized in eukaryotic cytosolic protein biosynthesis. In view of the absence of deformylase activity in mammalian cells, this is an attractive target for the design of novel antibiotic drugs. Co-crystallization of peptide deformylase from *Leptospira interrogans* (*Li*PDF) with its natural inhibitor actinonin produced diffraction-quality crystals that belong to space group $P2_1$, with unit-cell parameters a = 87.5, b = 119.1, c = 95.8 Å, β = 111.6°. The 3.1 Å resolution data set collected in-house was used to obtain phases by molecular replacement. Three schemes for the correction of the preliminary solutions were proposed and proved successful in determining the structure of *Li*PDF with eight subunits in the asymmetric unit.

1. Introduction

A distinctive feature of protein synthesis in prokaryotes is that translation initiation involves an additional step of formylation of methionyl-tRNAmet (Meinnel & Blanquet, 1995). In fact, together with a specialized tRNA, methionine plays a key role in the translation-initiation step in all organisms. This process involves the addition of a formyl group onto Met-tRNA^{fMet} molecules in prokaryotes, mitochondria and chloroplasts (Meinnel *et al.*, 1997). Consequently, all nascent polypeptides synthesized in bacteria, mitochondria and chloroplasts bear an N-terminal formyl group (Bracchi-Ricard *et al.*, 2001). As a result, the subsequent deformylation of proteins catalyzed by peptide deformylase (PDF) is a prerequisite step for proper post-translational modification of eubacterial proteins and is therefore essential for growth. However, the formylation/deformylation pathway is not a general feature of protein synthesis in higher organisms, although deformylase genes have been identified in the cells of higher plants and humans (Madison *et al.*, 2002). In view of the absence of deformylase activity in mammalian cells, PDF appears to be a very attractive target in the search for new antibacterial agents against multi-resistant bacteria and associated diseases.

PDF was first reported as an iron-containing enzyme that is very labile to assay conditions because of its conversion to Fe^{3+}(ferric ion) by molecular oxygen, resulting in inactivation (Manish *et al.*, 2002). Substitution of Ni^{2+} or Co^{2+} for the Fe^{2+} cofactor retains almost full catalytic activity, whereas substitution by Zn^{2+} reduces the activity by over two orders of magnitude (Bracchi-Ricard *et al.*, 2001). In contrast, recent work on the eukaryotic PDF1AS indicates it to be a zinc-containing enzyme with full activity (Serero *et al.*, 2001). These findings suggest a more complex situation than was initially thought.

Here, we report the co-crystallization of actinonin, a naturally occurring antibacterial agent, with peptide deformylase from *Leptospira interrogans* (*Li*PDF), a ubiquitous environmental bacterium causing acute febrile illness. According to our previous data (Li, Chen *et al.*, 2002), *Li*PDF is a fully active zinc-containing enzyme with high specificity. In addition, its dimeric association in solution was observed to be very stable, while all other PDFs purified so far were reported to be monomers. Here, we present the preliminary crystallographic analysis of *Li*PDF complexed with actinonin and molecular-replacement schemes with eight subunits in the asymmetric unit.

2. Purification

The zinc-containing *Li*PDF was expressed in 2 l LB medium according to the standard method described by Sambrook & Russell (2001) and was purified basically following the protocol reported previously (Li, Ren *et al.*, 2002). The pH value of the buffer used in purification was adjusted to 7.5 and no DTT was added. A linear gradient of 10–500 m*M* NaCl was used to elute the DEAE Sepharose

column. Fractions containing PDF were concentrated and applied to a S-200 size-exclusion column. However, the pooled protein was still not sufficiently pure for crystallization at this stage. It was therefore further purified using a high-performance Mono-Q column. Target fractions were collected and prepared for crystallization with a final concentration of approximately 30 mg ml^{-1}.

3. Co-crystallization and data collection

The enzyme was co-crystallized with the natural inhibitor actinonin using the hanging-drop vapour-diffusion method at 277 K. Sparse-matrix screening of 98 conditions from a Hampton crystallization kit (Jancarik & Kim, 1991) was carried out with droplets containing 1 µl of protein solution (30 mg ml^{-1} protein in 50 m*M* Tris–HCl pH 7.5, 10 m*M* NaCl), 1 µl actinonin solution (the final molar concentration of actinonin is 100 times higher than that of *Li*PDF after mixing) and 1 µl well solution. Several conditions produce crystals of suitable size. However, almost all of the crystals failed to diffract well enough for structure determination. Finally, only condition 57, which contains 10%(*w*/*v*) PEG 1K and 10%(*w*/*v*) PEG 8K, produced crystals that were good enough for diffraction. Subsequent PEG-concentration screening was performed in order to optimize this condition. Interestingly, crystals were easily obtained but only a few of them were usable for diffraction; others from the same crystallization solution, even from the same drop, hardly diffracted.

Data were collected on a MAR Research image-plate system with a local 2.0 kW X-ray source at room temperature. The wavelength was 1.5418 Å and the exposure time was 20 min per image. The oscillation step for each image was 1°. The crystal-to-detector distance was set to 200 mm. The data were processed with *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The self-rotation function was calculated using the *CNS* package (Brünger *et al.*, 1998).

4. Molecular-replacement schemes

The twofold screw axis of the space group was identified by the systematic absences. The unit-cell parameters were a = 87.5, b = 119.1, c = 95.8 Å, β = 111.57° (Table 1). The V_M value is reasonable (3.27–1.76 Å^3 Da^{-1}), assuming the number of molecules in the asymmetric unit (AU) to be between 7 and 13. Calculation of the self-rotation function showed one crystallographic twofold symmetry axis with ψ = 0.0, φ = 0.0, κ = 180.0° and one non-crystallographic twofold symmetry axis with ψ = 90.0, φ = 69.5, κ = 177.7°. Together with the fact that *Li*PDF is associated as a dimer in solution (Li, Chen *et al.*, 2002), this analysis strongly suggests that the number of molecules in the AU is very likely to be eight, ten or 12. Subsequent molecular replacement with ten subunits in the AU was carried out using the *AMoRe* package (Navaza, 1994) with the native *Li*PDF structure (to be published elsewhere) as the initial model and produced an apparently good solution with a correlation coefficient of 0.529 and an R factor of 0.403. However, packing analysis using the program *O* (Jones *et al.*, 1991) showed that seven subunits of the ten were intertwined with each other. Interestingly, a similar situation occurred in the case of eight or 12 subunits in the AU. After many trials, this problem was overcome using three schemes as follows.

In the early attempts to correct the solutions described above, we deleted three subunits from the ten, keeping the remaining seven molecules separated from each other. Subsequent refinement of the modified solution was performed in *CNS* using standard protocols. Unexpectedly, annealing refinement followed by energy minimization reduced the values of R and $R_{\rm free}$ to 0.321 and 0.348, respectively, indicating a correct model for *Li*PDF. At this stage, a lump of $F_o - F_c$ density with a shape of a complete subunit appeared in the map, indicating the existence of the eighth molecule. This was confirmed by further refinement, into which another subunit was added manually. Finally, the model was proved to contain eight subunits, each of which was complexed with one molecule of actinonin.

In the second scheme, examination of the crystal packing and symmetry revealed that the preliminary solutions given by *AMoRe* contained a twofold-related dimer associated by hydrophobic interactions. Supposing that this dimer is biologically stable and could be retained as in the native PDF crystals, molecular replacement was therefore carried out with a starting model of a dimer of native PDF. As expected, four dimers were found in the correct solution. In addition, it should be noted that some protein tends to aggregate in oligomers of an even number of subunits. In this situation, molecular replacement can consequently be repeated with the oligomer found in the previous cycle as the starting model in turn and with the number of molecules in the AU reduced by half in each cycle. In our example, two tetramers were successfully identified to form an octamer at the end of the third cycle.

Table 1
Statistics of data collection and processing.

Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 87.5, b = 119.1, c = 95.8, β = 111.57
Resolution (Å)	20–3.1
No. reflections	183806
No. independent reflections	31226
Completeness (%)	94.1
$R_{\rm sym}$ (3.1 Å)	0.249
$I/\sigma(I)$	2.6

5. Discussion

The emergence of bacterial pathogens that are resistant to multiple classes of existing antibiotics has created an urgent demand for new antibacterial agents with novel mechanisms of action. Peptide deformylase is currently being pursued as an attractive target protein. However, although several structures of PDF have been determined by X-ray diffraction and NMR spectroscopy (Guilloteau *et al.*, 2002; Kumar *et al.*, 2002; Baldwin *et al.*, 2002; Chan *et al.*, 1997), the catalytic mechanism proposed by Becker *et al.* (1998) is not unified with that proposed by Chan *et al.* (1997). In addition, the disparity in catalytic efficiency with different metal ions has not been well elucidated on a structural level.

Study of the structural basis of inhibitor binding to PDF is of great importance for the design of inhibitors that are selective for microbes. To this end, we co-crystallized the peptide deformylase from the pathogen *L. interrogans* with the inhibitor actinonin. The subsequent crystallographic analysis revealed a highly conserved catalytic core around the metal ion and identified a stable biological dimer, in keeping with our previous findings that *Li*PDF is a homodimer in solution (Li, Chen *et al.*, 2002). To date, dimeric association has not been observed in other reported PDFs. The physiological significance of such a dimer in *L. interrogans* remains to be established.

The crystals of *Li*PDF complexed with actinonin only diffracted to 3.1 Å with a relatively high $R_{\rm sym}$ value, indicating poorly ordered crystal packing resulting from too many molecules in the unit cell. A simple explanation for such a situation lies in the flexibility of some loops, which needs to be confirmed by higher resolution diffraction data. Nevertheless, owing to the eightfold non-crystallographic symmetry redundancy,

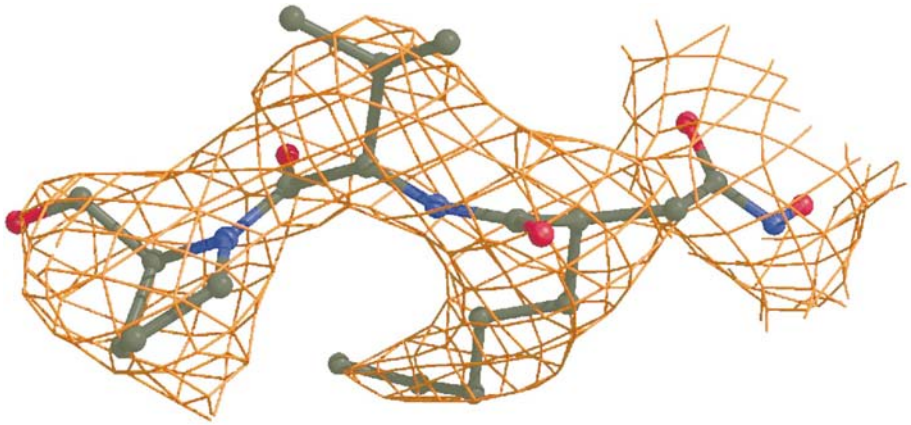

Figure 1
Electron density in the active pocket calculated from ($F_o - F_c$) maps in the early refinement. The inhibitor actinonin (rendered as ball and stick) was modelled according to this map. This figure was generated with *BOBSCRIPT* (Esnouf, 1997) and rendered by *RASTER3D* (Merritt & Bacon, 1997).

the resulting $2F_o - F_c$ and $F_o - F_c$ electron density is good enough for the assignment of the actinonin molecule (Fig. 1). Some conformational variations occurring in the active binding pocket were identified and compared with those of *Escherichia coli* PDF (details to be published elsewhere). In addition, three molecular-replacement schemes have been proposed for the case in which multiple monomers are involved. Further experiments with the aim of obtaining high-resolution diffraction are ongoing in our laboratory and seem promising.

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