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Correspondence e-mail: melissa.s.harris@pharmacia.com In bacteria the biosynthesis of all nascent polypeptides begins with Nformylmethionine. The post-translational removal of the N-formyl group is carried out by peptide deformylase (PDF). Processing of the N-formyl group from critical bacterial proteins is required for cell survival. This formylation/deformylation cycle is unique to eubacteria and is not utilized in eucaryotic cytosolic protein biosynthesis. Thus, inhibition of PDF would halt bacterial growth, spare host cellfunction, and would be a novel mechanism for a new class of antibiotic. Diffraction-quality Se-met crystals of S. aureus PDF were prepared that belong to space group  $C222_1$  with unit cell parameters of  $a = 94.1 \ b = 121.9 \ c = 47.6 \ \text{Å}$ . Multiple anomalous dispersion data were collected at the Advanced Photon Source 17-ID beamline and used to solve the PDF structure to 1.9 Å resolution. Crystals were also prepared with three PDF inhibitors: thiorphan, actinonin and PNU-172550. The thiorphan and actinonin co-crystals belong to space group C222<sub>1</sub> with similar unit-cell dimensions. Repeated attempts to generate a complex structure of PDF with PNU-172550 from the orthorhombic space group were unsuccessful. Crystallization screening identified an alternate C2 crystal form with unitcell dimensions of  $a = 93.4 \ b = 42.5 \ c = 104.1 \ \text{\AA}, \ \beta = 93^{\circ}$ .

# Co-crystallization of *Staphylococcus aureus* peptide deformylase (PDF) with potent inhibitors

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#### 1. Introduction

Initiation of translation in eubacteria utilizes formylmethionine-charged -tRNA for the ribosome-mediated biosynthesis of the first peptide bond. As a result, all nascent polypeptides are synthesized with N-formyl methionine at the N-terminus (Meinnel & Blanquet, 1995). The formyl group is removed by peptide deformylase (PDF) during elongation or shortly after completion of polypeptide biosynthesis (Adams, 1968; Ball & Kaesberg, 1973; Livingston & Leder, 1969). Removal of the formyl group is essential to cell survival and it has been shown that deletion of the PDF gene in Escherichia coli is lethal (Mazel et al., 1994; Clements et al., 2001). This formylation/ deformylation cycle is unique to eubacteria and is not utilized in eukaryotic cytosolic protein biosynthesis. The deformylation activity of PDF is an attractive target for a potential new class of antibiotics. A structurebased drug-design effort was initiated to support the elaboration of specific PDF inhibitors. Ligand-free PDF crystals and three complexes of PDF with potent ligands from the human pathogen Staphylococcus aureus were prepared and X-ray diffraction data were collected to beyond 2.0 Å resolution.

#### 2. Experimental procedures

#### 2.1. Expression and purification

The S. aureus peptide deformylase sequence was identified, cloned and purified as previously described (Baldwin et al., 2002). The gene encodes a total of 189 residues, including a C-terminal hexahistadine tag. The molecular weight of the PDF protein is 19 kDa. Protein was provided for crystallization in 25 mM Tris-HCl pH 8.0 and 50 mM NaCl at a protein concentration between 30 and  $60 \text{ mg ml}^{-1}$ . Protein samples were diluted to  $30 \text{ mg ml}^{-1}$ with matching buffer solution, snap-frozen in liquid nitrogen and stored at 193 K for subsequent crystallization experiments. Selenomethionine-enriched PDF was also expressed and purified for crystallization experiments. Incorporation of SeMet was accomplished through the down-regulation of methionine biosynthesis just prior to induction of PDF expression with isopropyl  $\beta$ -D-1-thiogalactopyranoside (Benson et al., 1995; Van Duyne et al., 1993).

#### 2.2. Ligand-free crystallization

Sparse-matrix crystallization screens of ligand-free PDF were set up in 2  $\mu l$  hanging

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drops at 293 K (Jancarik & Kim, 1991). After 14 d, long thin needle clusters were observed from Hampton Crystal Screen condition No. 6 (30% PEG 4000, 0.2 M MgCl<sub>2</sub> and 0.1 M Tris pH 8.5) (Fig. 1). Gridscreen optimization experiments varying PEG 4000 and MgCl<sub>2</sub> concentrations, pH and drop size continued to produce thin rodshaped clusters. Single rod-shaped crystals were finally achieved by microseeding 8 µl sitting drops at set up with a  $10^{-3}$  diluted seed stock using a cat's whisker. Seed-stock dilutions were prepared by crushing needle clusters in stabilization solution (25% PEG 4000, 0.2 M MgCl<sub>2</sub> and 0.05 M Tris pH 8.5) using the seed bead kit (Hampton Research). A crystal measuring  $0.22 \times 0.20$  $\times$  0.60 mm grew from 20% PEG 4000, 0.1 M MgCl<sub>2</sub> and 0.05 M Tris pH 8.5 (Fig. 1). Crystals were cryoprotected over a 2 h time period by slowly adding increasing cryosolution (25% PEG 4000, 0.1 M MgCl<sub>2</sub>, 0.05 M Tris pH 8.5 and 25% glycerol) directly to the drops. Crystals were cryocooled in liquid nitrogen and stored for cryogenic data collection. SeMet-enriched protein crystals were also prepared by the method described.

## 2.3. Co-crystallization with potent inhibitors

Three inhibitors were selected for cocrystallization experiments with *S. aureus* PDF (Fig. 2). Two previously reported PDF inhibitors were chosen: actinonin (IC<sub>50</sub> = 3 n*M*) and thiorphan (IC<sub>50</sub> = 12  $\mu$ *M*) (Chen *et al.*, 2000; Clements *et al.*, 2001; Meinnel *et al.*, 1999). The third inhibitor, PNU-172550, with IC<sub>50</sub> = 27 n*M*, was discovered during high-throughput screening of a portion of the Pharmacia compound collection using the Vibrio aminopeptidase assay previously described in Baldwin *et al.* (2002).

PDF protein was mixed with 5 mMthiorphan, 2 mM actinonin or 5 mM PNU-172550 and incubated on ice for 1 h. All inhibitor stock solutions were prepared at 100 mM in 100% dimethyl sulfoxide. Sittingdrop vapour-diffusion experiments were set up by mixing 4 ul protein and 4 ul well solution containing 17-28% PEG 4000, 0.1 M MgCl<sub>2</sub> and 0.05 M Tris pH 8.5. After one to two weeks, heavy precipitation and needle clusters were observed at the higher PEG 4000 concentrations, but single crystals did not grow spontaneously. Single rodshaped crystals were achieved with all three ligands as a result of microseeding with a  $10^{-4}$  diluted ligand-free seed stock 1 d after setup. Single crystals grew within two weeks

#### Table 1

Data-collection parameters and preliminary processing results for the low-energy wavelength SeMet data set and each of the three complex data sets.

Values in parentheses are for the outermost resolution shell.

	SeMet (low)	Thiorphan	Actinonin	PHA-17255
Wavelength (Å)	1.0332	1.0000	1.0000	1.0000
Crystal-to-detector distance (cm)	15.0	15.0	12.0	14.0
Exposure time (s)	0.5	1.0	2.0	1.0
No. of frames	300	300	120	600
Frame width (°)	0.25	0.25	1.00	0.50
Space group	C222 <sub>1</sub>	C222 <sub>1</sub>	C222 <sub>1</sub>	C2
Unit-cell parameters (Å)	-	-	-	
a (Å)	94.1	94.1	94.1	93.4
b (Å)	121.9	121.9	121.9	42.5
c (Å)	47.6	47.6	47.6	104.1
γ (°)				93
Resolution range (Å)	20.0-1.8	20.0-1.5	20.0-2.0	20.0-1.9
No. of observations	96952	70184	83619	191091
No. of unique reflections	21188	36628	17489	32247
Completeness (%)	97.5	85.3 (25.8)	96.7 (77.4)	99.4 (95.7)
$R_{\rm sym}^{\dagger}$ (%)	4.2 (10.7)	5.1 (20.9)	3.5 (16.2)	3.8 (18.9)
$\langle I/\sigma(I) \rangle$	31.6 (8.0)	30.0 (1.43)	30.0 (6.9)	49.7 (7.0)
Mosaicity (°)	0.53	0.573	0.473	0.59

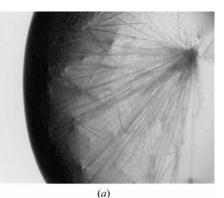
 $\dagger R_{\text{sym}} = (\sum |I - \langle I \rangle|) / \sum \langle I \rangle$ , where I is the observed intensity and  $\langle I \rangle$  is the average intensity of symmetry-related reflections.

(Fig. 2). Crystals were cryopreserved as described for the ligand-free crystals in 25% PEG 4000, 0.1 *M* MgCl<sub>2</sub>, 0.05 *M* Tris pH 8.5, 25% glycerol and 2–5 m*M* ligand.

Several attempts to obtain a complex structure of PDF and PNU-172550 from the above crystal form proved unsuccessful. Sparse-matrix screening using PDF  $(15 \text{ mg ml}^{-1})$  incubated with 1 mM PNU-172550 was initiated to identify a novel crystal form more amenable to PNU-172550 binding. Emerald Biostructures Wizard Screen 1 condition No. 6 (20% PEG 3000 and 0.1 M citrate pH 5.5) produced small plate clusters. Larger plate clusters were obtained by lowering the precipitant concentration, setting up larger 8 µl drops and converting to sitting-drop vapour diffusion. Crystals grew to up to 0.2  $\times$  0.1  $\times$ 0.03 mm in 5-10 d from 14% PEG 3000 and 0.1 M sodium citrate pH 5.5 (Fig. 2). Plates were cryopreserved in 14% PEG 3000, 0.1 M sodium citrate pH 5.5, 30% glycerol and 5 mM PNU-172550 by slow addition of the cryoprotectant directly to the drop. Individual plates were then teased from the cluster and cryocooled in liquid nitrogen for cryogenic data collection.

#### 2.4. Data collection and processing

Data were collected using synchrotron radiation at the Industrial Macromolecular Crystallography Association Collaborative Access Team (IMCA-CAT), beamline 17-ID at the Advanced Photon Source, Argonne National Laboratory. The ring current varied between 100 and 67 mA and the data sets were collected at 100 K using an Oxford Cryosystems Cryostream. The SeMet data



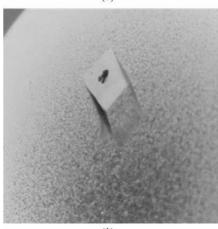
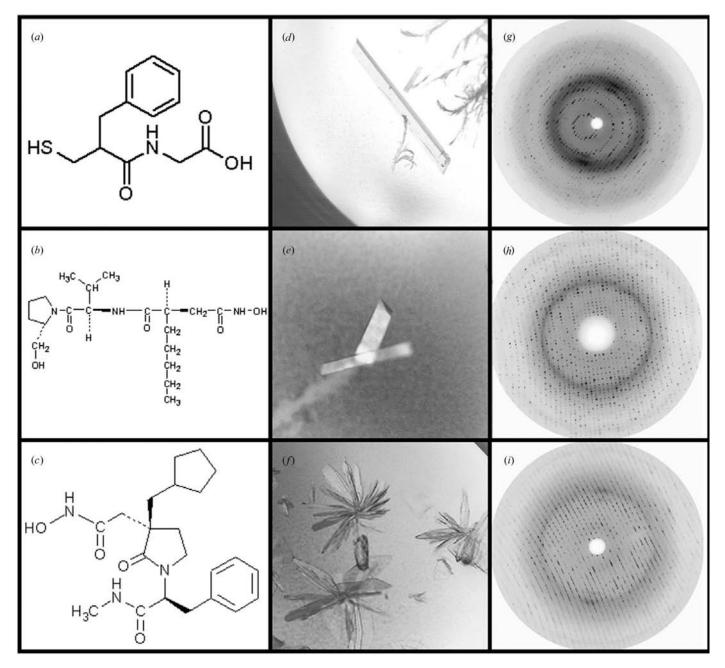




Figure 1 (*a*) Needle clusters of SeMet PDF were observed after two weeks from Hampton Crystal Screen condition No. 6. (*b*) Single rod-shaped crystals measuring  $0.35 \times 0.35 \times 0.70$  mm were finally achieved by microseeding.

sets were collected at three wavelengths (1.0332, 0.97939, 0.97928 Å) corresponding to the low-energy wavelength, inflection-point wavelength and the peak wavelength about the Se  $\kappa$  absorption edge. PDF–inhi-

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#### Figure 2

Three ligands were selected for co-crystallization experiments with PDF: (a) thiorphan, (b) actinonin and (c) PNU-172550. Co-crystals of PDF complexed with the three inhibitors are shown in (d) for thiorphan ( $0.08 \times 0.08 \times 0.37$  mm), (e) for actinonin ( $0.15 \times 0.10 \times 0.53$  mm) and (f) for PNU-172550 ( $0.2 \times 0.10 \times 0.03$  mm). Single diffraction images from each co-crystal are shown in (g), (h) and (i), respectively.

bitor complex data sets were collected at 1.000 Å. The SeMet-enriched PDF and thiorphan co-crystal data were collected using a Brüker CCD detector mounted upon a  $\kappa$  goniometer and the diffraction data were acquired and processed with *SAINT* (Siemens, 1993). The actinonin and PNU-172550 data sets were collected using a MAR CCD detector with *marccd* data-acquisition software (MAR, USA; M. Blum, unpublished work). Two-dimensional images were integrated and scaled with *HKL*2000 (Otwinowski & Minor, 1997).

Data-collection parameters and processing statistics for the low-energy wavelength SeMet PDF data set and each of the three ligand-complex data sets are shown in Table 1.

#### 3. Results and discussion

Analysis of data from the ligand-free SeMet PDF crystal indicates that it belongs to space group  $C222_1$ , with unit-cell parameters a = 94.1, b = 121.9, c = 47.6 Å. The crystal diffracts to 1.9 Å resolution and the ligand-

free crystal structure was successfully phased using the multiple anomalous dispersion data; a detailed description of this structure is reported elsewhere (PDB code 1lmh; Baldwin *et al.*, 2002). Generation of single PDF–inhibitor co-crystals using the cross-microseeding method with the ligandfree seeds proved very successful. The thiorphan and actinonin co-crystals also belong to space group  $C222_1$ , with similar unit-cell parameters. The co-crystals diffract strongly to 1.8 and 2.0 Å. There is one molecule per asymmetric unit in the  $C222_1$  crystals and the specific volume  $V_{\rm M}$  is  $3.585 \text{ Å}^3 \text{ Da}^{-1}$  with a solvent content of 65.7% (Matthews, 1968). Difference Fourier maps using data from the  $C222_1$  co-crystals confirmed the presence of thiorphan and actinonin in the PDF active site. Attempts to obtain a complex structure of PDF with PNU-172550 in the orthorhombic space group failed repeatedly. This was surprising given the inhibitor's potent binding, good solubility and our observation of another hydroxamate inhibitor, actinonin, in a structure belonging to the  $C222_1$  space group. Analysis of the 1.9 Å resolution data generated from the PDF-PNU-172550 plate co-crystal reveals it to belong to space group C2, with unit-cell parameters a = 93.4,  $b = 42.5, c = 104.1 \text{ Å}, \gamma = 93^{\circ}$ . There are two molecules per asymmetric unit in the C2 cocrystal, with a specific volume of 2.69  $\text{\AA}^3$  Da<sup>-1</sup> and a solvent content of 54.3% (Matthews, 1968). The complex structure, solved by molecular replacement, reveals the binding mode of PNU-172550 and will be described elsewhere.

These crystallization experiments have established routine methods for the preparation of *S. aureus* PDF crystals with inhibitors that diffract to beyond 2.0 Å resolution. We have found that microseeding greatly increases both the speed and

success rate for PDF co-crystallization. Finally, we note that not all validated inhibitors will co-crystallize in the  $C222_1$  crystal form. However, novel crystallization conditions can be obtained that produce crystal forms, such as C2, that are more favourable for inhibitor binding.

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