Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Purification, crystallization and preliminary X-ray diffraction analysis of Cif, a virulence factor secreted by *Pseudomonas aeruginosa*

Christopher D. Bahl,[a] Daniel P. MacEachran,[b] George A. O'Toole[b] and Dean R. Madden[a]*

[a]Department of Biochemistry, Dartmouth Medical School, 7200 Vail Building, Hanover, NH 03755, USA, and [b]Department of Microbiology and Immunology, Dartmouth Medical School, 7550 Vail Building, Hanover, NH 03755, USA

Correspondence e-mail: drm0001@dartmouth.edu

Received 10 October 2009
Accepted 11 November 2009

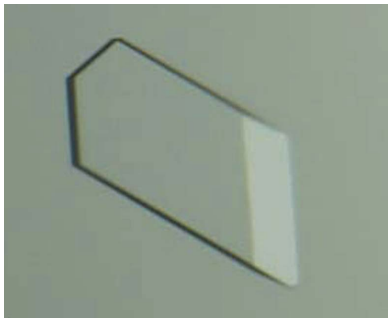
The opportunistic pathogen *Pseudomonas aeruginosa* secretes a protein that triggers the accelerated degradation of the cystic fibrosis transmembrane conductance regulator (CFTR) in airway epithelial cells. This protein, which is known as the CFTR inhibitory factor (Cif), acts as a virulence factor and may facilitate airway colonization by *P. aeruginosa*. Based on sequence similarity Cif appears to be an epoxide hydrolase (EH), but it lacks several of the conserved features found in the active sites of canonical members of the EH family. Here, the crystallization of purified recombinant Cif by vapor diffusion is reported. The crystals formed in space group $C2$, with unit-cell parameters a = 167.4, b = 83.6, c = 88.3 Å, β = 100.6°. The crystals diffracted to 2.39 Å resolution on a rotating-anode source. Based on the calculated Matthews coefficient (2.2 Å^3 Da^{-1}), it appears that the asymmetric unit contains four molecules.

1. Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium that is commonly found in soil. However, it is also an opportunistic pathogen that infects humans and forms biofilms that can become highly resistant to antibiotics (Davies & Bilton, 2009). *P. aeruginosa* commonly causes lung infections in patients with chronic obstructive pulmonary disease (Dudley *et al.*, 2008), ventilator-associated pneumonia (Luna *et al.*, 2009) and community-acquired pneumonia (Koulenti & Rello, 2006). Additionally, nearly 80% of cystic fibrosis patients develop chronic pulmonary *P. aeruginosa* infections by the time they reach the age of 18 (Geller, 2009).

Recently, it has been shown that *P. aeruginosa* can inhibit the cell-surface expression of cystic fibrosis transmembrane conductance regulator (CFTR) in human airway epithelial cells in culture (Swiatecka-Urban *et al.*, 2006). This activity was traced to a single secreted protein, dubbed the CFTR inhibitory factor (Cif; MacEachran *et al.*, 2007). CFTR activity is essential for the maintenance of mucociliary clearance mechanisms in the lung. By subverting CFTR expression, Cif may facilitate airway colonization by *P. aeruginosa*. In patients with cystic fibrosis, Cif may interfere with therapeutic efforts to restore CFTR function.

Based on sequence analysis, Cif appears to belong to the α/β hydrolase family (Ollis *et al.*, 1992), a group of proteins with a wide range of enzymatic activities. More specifically, it has been proposed that Cif is a member of the soluble epoxide hydrolase (EH) family (MacEachran *et al.*, 2007). However, the sequence identity underlying this assignment is low (<30% to confirmed EH members), and a number of residues that form the canonical EH active site exhibit sequence substitutions. Crystallographic data should help to clarify the status of Cif as an epoxide hydrolase and provide a basis for mutagenetic analysis of its CFTR inhibitory action *in vivo*.



2. Materials and methods

2.1. Protein expression and purification

The plasmid pDPM73 (MacEachran *et al.*, 2007) expresses full-length Cif, locus PA14_26090 from *P. aeruginosa* strain PA14 (Uni-

Prot accession No. Q02P97), which corresponds to locus PA2934 in strain PA01. The coding sequence is fused directly to a C-terminal hexahistidine tag, and the fusion protein is expressed under the control of a pBAD arabinose-inducible promoter. The accuracy of the construct was verified by DNA sequencing. Residue numbers are reported with respect to the full-length sequence.

Escherichia coli Top10 cells (Invitrogen) were transformed with pDPM73. Single colonies were inoculated and grown overnight in 10 ml lysogeny broth (LB; Bertani, 1951) containing 100 µg ml^{-1} ampicillin. Overnight cultures were then diluted 1:100 into LB supplemented with 100 µg ml^{-1} ampicillin and 0.2%(*w*/*v*) L-arabinose and incubated at 310 K with shaking for up to 48 h. Bacterial cells were removed from the medium by centrifugation at 12 000*g* for 20 min at 277 K followed by filtration through a 0.22 µm pore polyethersulfone membrane (Nalgene) at room temperature.

The filtered supernatant was adjusted to a final concentration of 5 m*M* imidazole and then passed over a 10 ml column of Ni–NTA Superflow resin (Qiagen) pre-equilibrated with IMAC buffer (500 m*M* NaCl, 20 m*M* sodium phosphate pH 7.4) containing 5 m*M* imidazole. The column was subsequently washed with 200 ml IMAC buffer containing 5 m*M* imidazole to remove unbound material. Cif was eluted from the column over a five-column-volume gradient running from 5 to 500 m*M* imidazole in IMAC buffer. Fractions were pooled and concentrated using an Amicon Ultra 15 centrifugation filter with a 10 000 molecular-weight cutoff as per the manufacturer's directions. The protein was then dialyzed against 20 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4, 100 m*M* NaCl or 20 m*M* sodium phosphate pH 7.4, 100 m*M* NaCl. Quantitative amino-acid analysis to determine a reference protein concentration and N-terminal sequence analysis by Edman degradation (Niall, 1973) were performed at the W. M. Keck Biotechnology Resource Laboratory (Yale University) using purified Cif protein. The $A_{280}^{1\,\mathrm{cm}}$ was measured for an aliquot of the same sample and used to calculate an experimental molar extinction coefficient of $\varepsilon = 47\,556\ M^{-1}\ \mathrm{cm}^{-1}$ (the calculated value was 46 995 $M^{-1}\ \mathrm{cm}^{-1}$). For routine measurements, the protein concentration was determined by Bradford assay (Bio-Rad) or by using the A_{280} and the Beer–Lambert law $A = \varepsilon cl$.

2.2. Crystallization of Cif

Purified Cif protein at 5 mg ml^{-1} in 20 m*M* HEPES pH 7.4, 100 m*M* NaCl was submitted to the Hauptman–Woodward Medical Research Institute High-Throughput Screening Facility for microbatch crystallization (Luft *et al.*, 2003). Vapor-diffusion crystallization was carried out by mixing purified Cif in a 1:1 ratio with reservoir solution in 2 µl sitting drops or 4 µl hanging drops and incubating at 291 K. For sitting-drop experiments, protein was prepared at 5 mg ml^{-1} in 20 m*M* HEPES pH 7.4, 100 m*M* NaCl. For hanging-drop experiments, protein was prepared at 5 mg ml^{-1} in 20 m*M* sodium phosphate pH 7.4, 100 m*M* NaCl. A 100 µl reservoir was used for sitting drops and a 400 µl reservoir was used for hanging drops. The reservoir solution yielding the crystal used for X-ray diffraction consisted of 20%(*w*/*v*) polyethylene glycol (PEG) 8000, 100 m*M* $CaCl_2$, 100 m*M* sodium acetate pH 5 in a sitting drop.

2.3. Data collection

Prior to data collection, a crystal was transferred into cryoprotectant buffer [20%(*w*/*v*) PEG 8000, 100 m*M* $CaCl_2$, 100 m*M* sodium acetate pH 5, 20%(*w*/*v*) glycerol] and flash-cooled in the nitrogen-gas stream of an Oxford Cryostream 700 operating at 100 K. Diffraction data were collected at 100 K on a MAR345dtb image-plate system (Rayonix) using Cu $K\alpha$ radiation from a rotating-anode generator (Rigaku) equipped with focusing optics (Genova).

A data set was obtained by collecting 600 oscillation images of 0.3° each over a total of 180°, with an exposure time of 120 s per image.

Table 1
Crystallographic and data-collection statistics.

Values in parentheses are for the highest resolution shell.

Space group	*C*2
Unit-cell parameters (Å, °)	a = 167.4, b = 83.6, c = 88.3, α = 90, β = 100.6, γ = 90
No. of monomers per ASU	4
Matthews coefficient (Å^3 Da^{-1})	2.2
Solvent content (%)	44.7
Wavelength (Å)	1.54179
Resolution (Å)	19.67–2.39 (2.53–2.39)
No. of reflections	173567 (24613)
No. of unique reflections	47107 (7104)
R_{merge} (%)†	5.4 (9.5)
$\langle I/\sigma(I)\rangle$	16.3 (10.1)
Completeness (%)	99.1 (95.9)

† $R_{\mathrm{merge}} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ and $I(hkl)$ are the ith and the mean measurements of the intensity of reflection hkl.

2.4. Data analysis

Data were processed using the *XDS* package (Kabsch, 1993; Table 1). Self-rotation function analysis was carried out using *MOLREP* v.9.2 (Vagin & Teplyakov, 1997) as part of the *CCP*4 program suite v.6.0.2 (Collaborative Computational Project, Number 4, 1994). Molecular replacement was carried out using *PHENIX* v.1.3 (Adams *et al.*, 2002).

3. Results and discussion

Here, we present conditions for the expression and purification of the Cif protein from *P. aeruginosa*. The Cif protein contains a predicted secretory signal sequence from residues 1 to 24. Cleavage of the signal sequence following membrane translocation was confirmed by Edman degradation, which yielded the N-terminal sequence ^{25}AEEFPVPN. Highly purified mature hexahistidine-tagged Cif

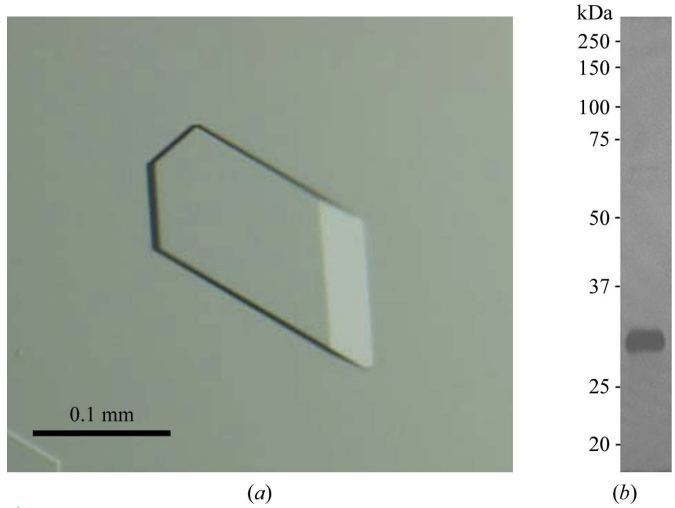


Figure 1
Crystallization of Cif. (*a*) A representative crystal of Cif obtained by hanging-drop vapor diffusion. The well solution contained 15%(*w*/*v*) PEG 8000, 125 m*M* $CaCl_2$ and 100 m*M* sodium acetate pH 5. (*b*) An SDS–PAGE gel of a washed Cif crystal, visualized by silver staining. A sample of mother liquor from the same drop was washed in an identical manner and gave no visible band (data not shown).

protein was obtained directly from the bacterial growth medium after sterilization by centrifugation and filtration. The bacteria were diluted 1:100 directly from an overnight culture into growth media containing L-arabinose to induce *cif*-gene expression. Cif protein is visible by Western blotting in the culture supernatant as early as 5 h post-induction, with maximal expression occurring at around 10 h (data not shown). The protein is stable at 310 K and between pH 6 and 9 and is poorly soluble at or below pH 4. Cif can be flash-frozen and stored at 193 K with 10%(*w*/*v*) glycerol added as a cryoprotectant.

In order to determine crystallization conditions for Cif, high-throughput screening of 1536 conditions by the microbatch-under-oil method was performed at the Hauptman–Woodward Institute (Luft *et al.*, 2003). Visual screening identified 12 conditions that yielded promising crystals, all of which included either acetate or citrate as a buffer, were between pH 4 and 5 and contained PEG as the precipitant. These conditions were reproduced by vapor diffusion (Fig. 1*a*). Although in many cases the precipitant, salt and/or protein concentrations identified by microbatch experiments need to be adjusted for use in vapor-diffusion experiments (Chayen, 1998), in this case we found that the best diffracting protein crystal came from vapor-diffusion experiments using the same conditions that were identified by microbatch screening.

To verify the identity of the crystallized protein, crystals were harvested, washed in well solution and dissolved in SDS–PAGE sample buffer. The protein from a washed crystal runs as a single band on SDS–PAGE at the expected molecular weight (Fig. 1*b*).

Glycerol was tested for its ability to support flash-cooling of the Cif crystallization buffer. 20%(*w*/*v*) glycerol was found to be sufficient to permit vitrification, and Cif crystals harvested into the corresponding cryobuffer exhibited excellent diffraction characteristics. A full data set was obtained on a rotating-anode source with a resolution limit of 2.39 Å ($R_{\rm merge}$ = 0.054; see Table 1).

Cif crystallizes in space group *C*2. Based on a calculated Matthews coefficient of 2.2 Å^3 Da^{-1}, it appears that the asymmetric unit contains four copies of the molecule. Self-rotation function analysis of these data using *MOLREP* revealed no significant fourfold non-crystallographic symmetry peaks, but did show a strong local twofold peak oriented along the crystallographic *a* axis (θ = 90°, φ = 180°, χ = 180.0°), with a score corresponding to 53% of the crystallographic peak. Additional twofold peaks are found with substantially lower scores (<16%). Since other bacterial α/β hydrolases form dimers (*e.g.* *Agrobacterium radiobacter* AD1 epoxide hydrolase; ArEH; Nardini *et al.*, 1999), it is possible that the crystals reported here contain two dimers in the asymmetric unit. Attempts to determine phase information by molecular replacement using ArEH (PDB entry 1ehy) as a search model failed to yield convincing solutions, presumably reflecting the low sequence identity (22%). Structure determination using experimental phases is under way and the refined structure will be reported separately.

Funding support was provided by the Cystic Fibrosis Foundation (MADDEN08G0 to DRM and STANTO97R0 Research Development Program pilot funds to GAO), the NIH (R01-DK075309 to DRM and training grant predoctoral fellowships T32-AI007519 to CDB and T32-DK007301 to DPM) and by a Rosalind Borison Memorial Predoctoral Fellowship to DPM.

References

Adams, P. D., Grosse-Kunstleve, R. W., Hung, L.-W., Ioerger, T. R., McCoy, A. J., Moriarty, N. W., Read, R. J., Sacchettini, J. C., Sauter, N. K. & Terwilliger, T. C. (2002). *Acta Cryst.* D**58**, 1948–1954.
Bertani, G. (1951). *J. Bacteriol.* **62**, 293–300.
Chayen, N. E. (1998). *Acta Cryst.* D**54**, 8–15.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Davies, J. C. & Bilton, D. (2009). *Respir. Care*, **54**, 628–640.
Dudley, M. N., Loutit, J. & Griffith, D. C. (2008). *Curr. Opin. Biotechnol.* **19**, 637–643.
Geller, D. E. (2009). *Respir. Care*, **54**, 658–670.
Kabsch, W. (1993). *J. Appl. Cryst.* **26**, 795–800.
Koulenti, D. & Rello, J. (2006). *Curr. Opin. Pulm. Med.* **12**, 198–204.
Luft, J. R., Collins, R. J., Fehrman, N. A., Lauricella, A. M., Veatch, C. K. & DeTitta, G. T. (2003). *J. Struct. Biol.* **142**, 170–179.
Luna, C. M., Sibila, O., Agusti, C. & Torres, A. (2009). *Eur. Respir. J.* **33**, 182–188.
MacEachran, D. P., Ye, S., Bomberger, J. M., Hogan, D. A., Swiatecka-Urban, A., Stanton, B. A. & O'Toole, G. A. (2007). *Infect. Immun.* **75**, 3902–3912.
Nardini, M., Ridder, I. S., Rozeboom, H. J., Kalk, K. H., Rink, R., Janssen, D. B. & Dijkstra, B. W. (1999). *J. Biol. Chem.* **274**, 14579–14586.
Niall, H. D. (1973). *Methods Enzymol.* **27**, 942–1010.
Ollis, D. L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S. M., Harel, M., Remington, S. J., Silman, I., Schrag, J., Sussman, J. L., Verschueren, K. H. G. & Goldman, A. (1992). *Protein Eng.* **5**, 197–211.
Swiatecka-Urban, A., Moreau-Marquis, S., Maceachran, D. P., Connolly, J. P., Stanton, C. R., Su, J. R., Barnaby, R., O'Toole, G. A. & Stanton, B. A. (2006). *Am. J. Physiol. Cell Physiol.* **290**, C862–C872.
Vagin, A. & Teplyakov, A. (1997). *J. Appl. Cryst.* **30**, 1022–1025.