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Expression, purification, crystallization and preliminary X-ray analysis of *Aeromonas hydrophilia* metallo-β-lactamase

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The CphA metallo-β-lactamase from *Aeromonas hydrophilia* has been expressed, purified and crystallized by the hanging-drop vapor-diffusion method using ammonium sulfate as the precipitant. The crystals exhibit orthorhombic symmetry ($P2_12_12$), with unit-cell parameters a = 40.75, b = 42.05, c = 128.88 Å. There is one monomer in the asymmetric unit and the solvent content is estimated to be 44% by volume. A data set extending to 1.8 Å has been measured.

1. Introduction

Metallo-β-lactamases have been identified as one mechanism for generating clinical resistance to antibiotics in pathogenic bacteria. There are four β-lactamase sequence families, termed classes A, B, C and D; the class A, C and D enzymes are active-site serine enzymes, while the class B enzymes depend on the presence of zinc for their activity. Currently, a non-redundant database (Galleni *et al.*, 2001) contains over 50 sequences of confirmed metallo-β-lactamases. The proteins vary in length between 240 and 310 amino-acid residues and contain signal peptides of 17–30 residues. Based on sequence similarity, the class B enzymes have been grouped into subclasses B1, B2 and B3 (Garau *et al.*, 2004; Galleni *et al.*, 2001).

The expression, purification and three-dimensional structures of three enzymes from subclass B1 (those from *Bacillus cereus*, *Bacteriodes fragilis* and *Pseudomonas aeruginosa*) and the *Stenotrophomonas maltophilia* enzyme from subclass B3 have been described. References to the many studies describing these structures can be found in a recent review of metallo-β-lactamases (Herzberg & Fitzgerald, 2004). The expression and purification of the CphA enzyme from *Aeromonas hydrophilia*, which belongs to subclass B2, has also been reported (Hernandez Valladares *et al.*, 1996), but to date there is no three-dimensional structure available for this subclass.

The *Aeromonas hydrophilia* CphA metallo-β-lactamase exhibits narrow substrate specificity (Felici *et al.*, 1993). It has been shown to be active in a mono-zinc form (Hernandez Valladares *et al.*, 1997), in contrast to the subclass B1 and B3 enzymes, which have two Zn atoms in the active site. Furthermore, it has been shown that the addition of a second Zn atom to CphA inhibits the enzyme activity (Hernandez Valladares *et al.*, 1997). To better understand these phenomena, we have undertaken structural studies of the CphA enzyme. Here, we report the expression, purification and crystallization of *A. hydrophilia* CphA and the preliminary crystallographic analysis of the crystals.

2. Protein expression and purification

A full-length clone of AE036 in pET-9a vector transformed into *Escherichia coli* HB101 was kindly provided by Professor G. Amicosante (Università degli Studi di L'Aquila, Italy). The plasmid was transformed into *E. coli* strain BL21 (DE3) pLysS for protein expression. A 50 ml overnight culture was grown from a colony and then diluted into 500 ml LB/kanamycin (20 ml overnight) and grown for 16 h at 310 K with no IPTG induction (expression tests with and without IPTG induction had shown no difference in protein yields).

Cells were lysed using the freeze–thaw method. The lysate was then loaded onto an SP-Sepharose FF column and the column was eluted with an NaCl gradient (0.0–0.3 *M*). The fractions containing imipenem-hydrolysing activity were pooled together and dialyzed against buffer containing no salt. The dialyzed material was loaded onto an iminodiacetic acid agarose (4% cross-linked, Affiland, Belgium) column that had been treated with 50 m*M* $ZnCl_2$, washed extensively with distilled water and equilibrated with 15 m*M* sodium cacodylate pH 6.5. The desired protein was eluted at 0.14 *M* NaCl and the fractions were concentrated and stored at 193 K. The final purified protein was homogeneous in SDS–PAGE.

3. Crystallization experiments

All crystallization reagents were obtained from Hampton Research, Laguna Niguel, CA, USA. For crystallization, the protein was concentrated to 9 mg ml^{-1}. Initial screening was performed using Hampton Research Crystal Screen I and Crystal Screen II at room temperature in hanging-drop vapor-diffusion plates. Each experiment utilized a 3 µl drop containing equal volumes of concentrated protein solution and screen solution. Each hanging drop was equilibrated against a 1 ml reservoir of screen solution. For the initial screening, the protein was in a buffer containing 15 m*M* cacodylate pH 7.0 and 140 m*M* NaCl. These experiments did not identify any crystals or promising leads. Thus, the protein was exchanged into a salt-free buffer and Hampton Research Crystal Screens I and II and PEG/Ion Screen were set up. Small crystals were obtained after several days in Crystal Screen I condition No. 20 (0.1 *M* sodium acetate pH 4.6, 25% PEG 4000, 0.2 *M* ammonium sulfate). Attempts to optimize condition No. 20 did not lead to diffraction-quality crystals.

We then attempted to crystallize the enzyme in complex with an inhibitor. The protein was incubated for 30 min on ice with a twofold molar excess of compound A (Fig. 1), prepared as a 20 m*M* stock in DMSO. Preliminary crystallization conditions were identified using Crystal Screen I with the hanging-drop vapor-diffusion method. Condition No. 4, containing 0.1 *M* Tris pH 8.5 and 2.0 *M* ammonium sulfate, produced a cluster of needles (Fig. 2*a*). Streak-seeding with these needles did not improve the size of the crystals.

While these studies were ongoing, we were also involved in a structure-determination effort involving the IMP-1 metallo-β-lactamase from *Pseudomonas aeruginosa* (Toney *et al.*, 2001). Very large IMP-1 crystals that diffracted to beyond 1.5 Å were obtained easily by streak-seeding. Given the robust nature of the IMP-1 crystal seeding system, we investigated the possibility of using IMP-1 crystals to seed nucleation of the CphA protein. A large irregular crystal (Fig. 2*b*) was obtained after three months incubation from one of these cross-seeded experiments. Given the long incubation time, it is not clear whether the cross-seeding influenced the eventual appearance of this crystal, but neither can this possibility be discounted. It is certainly true that no experiment that was not cross-seeded ever produced a large single crystal of the CphA protein. Treatment of the crystal shown in Fig. 2(*b*) with Izit dye (Hampton Research) demonstrated that the malformed crystal was indeed protein and the image shown in Fig. 2(*b*) is of the dye-treated crystal.

The availability of a single large crystal allowed propagation to improve the crystal quality. We successfully propagated the crystals using streak-seeding and found that quality of crystals obtained was time-dependent. Drops were equilibrated for 3 h, 6 h or overnight before seeding and we found that the overnight equilibration gave the largest and best formed crystals. We also tested a microseeding procedure (Fitzgerald & Madsen, 1986); a portion of the crystal

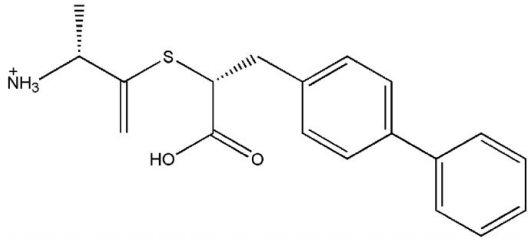
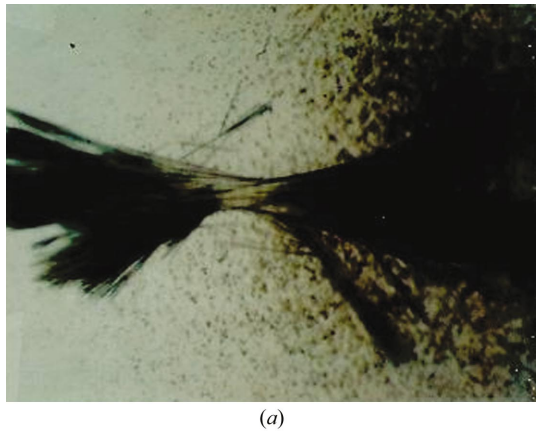
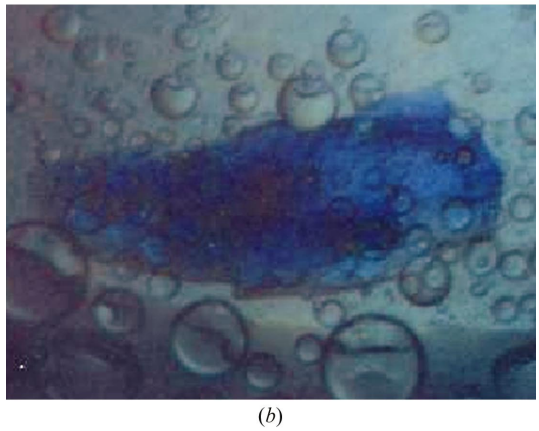


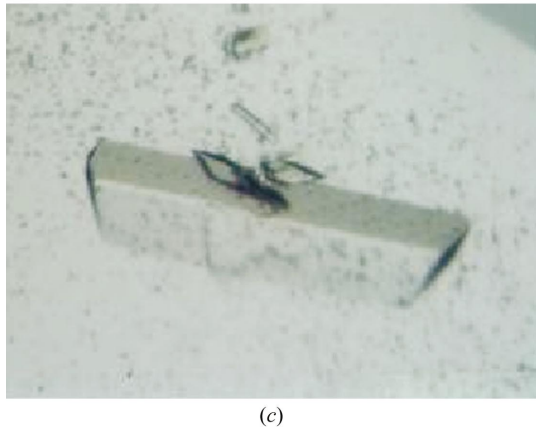
Figure 1
Chemical structure of compound A, the inhibitor used in co-crystallization experiments.



(*a*)



(*b*)



(*c*)

Figure 2
(*a*) Initial crystal of *A. hydrophilia* CphA in complex with compound A. (*b*) A large but badly formed crystal obtained by cross-seeding a CphA crystallization experiment with seeds obtained from crystals of the IMP-1 metallo-β-lactamase. (*c*) Final data-collection crystal of CphA.

Table 1
Crystal data-collection and data-processing statistics.

Values in parentheses are for the highest resolution shell.

Data collection	
Wavelength (Å)	1.0
Oscillation range (°)	1.0
Exposure time (s)	3.0
Rotation per image	1.0
Total rotation	150.0
Distance (cm)	12.5
Temperature (K)	100
Data processing	
Resolution limit	99.9–1.8 (1.91–1.80)
Total No. of measured reflections	107787 (19189)
No. of unique reflections	21312 (3497)
Completeness (%)	99.8 (100.0)
Redundancy	5.1 (5.5)
$R_{\rm merge}$	0.053 (0.141)
Mean $I/\sigma(I)$	61.3 (22.6)

shown in Fig. 2(*b*) was transferred into an Eppendorf tube containing seed bead (Hampton Research, Laguna Niguel CA, USA) and 100 µl Crystal Screen I reagent 4. The sample was vortexed for 1 min to generate a seed stock and ten serial dilutions (25 µl seed stock in 75 µl reservoir solution) were made before seeding (1 µl seed stock added to 3 µl drop). The microseeding procedure did yield crystals, but those crystals were not as good as those obtained by streak-seeding with overnight equilibration.

The resultant crystals were still not of the desired quality for data collection. Thus, we attempted to identify an additive that would improve the crystal quality. Drops containing 1 µl protein and 1 µl reservoir solution were equilibrated against 500 µl reservoir solution (0.1 *M* Tris pH 8.5, 2.0 *M* ammonium sulfate) and different additive screens (Hampton Research Additive Screens 1, 2 and 3) were tested using these conditions. Large diffraction-quality crystals (Fig. 2*c*) were obtained in the presence of 5 µl 50% Jeffamine M-600 pH 7.0 in the well. These crystals grew to maximum dimensions of 0.4 × 0.1 × 0.1 mm in two weeks.

4. Data collection and X-ray crystallographic analysis

All diffraction data were collected at beamline 17-ID of the Advanced Photon Source, Argonne National Laboratory, Argonne, IL, USA. Initially, a crystal was flash-cooled for data collection by direct exposure to a liquid-nitrogen stream. The diffraction pattern was strong, but it was contaminated with weaker patterns from small adhering crystals and with ice rings. The crystal was therefore removed from the cold stream and washed in a solution containing 10% glycerol in mother liquor. The crystal was then recooled, again by direct exposure to the liquid-nitrogen stream; the diffraction pattern was then free of ice rings and of secondary patterns from adhering crystals. A complete data set extending to 1.8 Å was measured and processed using the program *X-GEN* (Howard, 2001).

The unit-cell parameters were found to be a = 40.75, b = 42.05, c = 128.88 Å, with all unit-cell angles equal to 90°. Analysis of systematically absent reflections indicated the symmetry of space group $P2_12_12$. Volume considerations predicted one molecule in the asymmetric unit and a solvent content of 44%. The data collection and processing are described in Table 1.

Attempts were made to determine phases for this structure using the molecular-replacement technique. All known structures of metallo-β-lactamases were tested as molecular-replacement probes and none yielded a convincing solution. A search with a model containing a superposition of several varied metallo-β-lactamase structures did yield a molecular-replacement solution that packed properly in the crystal lattice. However, attempts to refine the structure beginning with this starting model have not been successful. Determination of this structure is likely to require the acquisition of experimental phase information.

5. Discussion

We have successfully expressed a full-length clone of *A. hydrophilia* CphA metallo-β-lactamase using *E. coli* as an expression host. The protein was purified to homogeneity using a two-step procedure and crystallized. This was a difficult protein to crystallize and success was achieved by employing both traditional and non-traditional crystallization techniques. The first large crystal that was obtained resulted from an experiment that had been cross-seeded with a different protein, the IMP-1 metallo-β-lactamase from *P. aeruginosa*. Once that crystal was identified, diffraction-quality crystals were eventually obtained using streak-seeding in the presence of the additive Jeffamine M-600.

Note added in proof: following acceptance of this manuscript, a report has been published describing the structure of CphA in a different space group (Garau *et al.*, 2005).

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References

Felici, A., Amicosante, G., Oratore, A., Strom, R., Ledent, P., Joris, B., Fanuel, L. & Frere, J. M. (1993). *Biochem. J.* **291**, 151–155.
Fitzgerald, P. M. D. & Madsen, N. B. (1986). *J. Cryst. Growth*, **76**, 600–606.
Garau, G., Brebone, C., Anne, C., Galleni, M., Frere, J.-M. & Dideberg, O. (2005). *J. Mol. Biol.* **345**, 785–795.
Garau, G., Garcia-Saez, I., Brebone, C., Anne, C., Mercuri, P., Galleni, M., Frere, J.-M. & Dideberg, O. (2004). *Antimicrob. Agents Chemother.* **48**, 2347–2349.
Galleni, M., Lamotte-Brasseur, J., Rossolini, G. M., Spencer, J., Dideberg, O. & Frere, J.-M. (2001). *Antimicrob. Agents Chemother.* **45**, 660–663.
Hernandez Valladares, M., Felici, A., Weber, G., Adolph, H. W., Zeppezauer, M., Rossolini, G. M., Amicosante, G., Frere, J.-M. & Galleni, M. (1997). *Biochemistry*, **36**, 11534–11541.
Hernandez Valladares, M., Galleni, M., Frere, J.-M., Felici, A., Perilli, M., Franceschini, N., Rossolini, G. M., Oratore, A. & Amicosante, G. (1996). *Microb. Drug Resist.* **2**, 253–256.
Herzberg, O. & Fitzgerald, P. M. D. (2004). *Handbook of Metalloproteins*, Vol. 3, edited by A. Messerschmidt, W. Bode & M. Cygler, pp. 217–234. Chichester: John Wiley & Sons.
Howard, A. J. (2001). *Crystallographic Computing 7: Proceedings of the Macromolecular Computing School, 1996*, edited by P. E. Bourne & K. D. Watenpaugh, pp. 150–165. Oxford University Press.
Toney, J. H., Hammond, G. G., Fitzgerald, P. M., Sharma, N., Balkovec, J. M., Rouen, G. P., Olson, S. H., Hammond, M. L., Greenlee, M. L. & Gao, Y. D. (2001). *J. Biol. Chem.* **276**, 31913–31918.