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Crystal Structures of *Pseudomonas aeruginosa* GIM-1: Active-Site Plasticity in Metallo-β-Lactamas

Pardha Saradhi Borra, Ørjan Samuelsen, James Spencer, Timothy R. Walsh, Marit Sjo Lorentzen, Hanna-Kirsti S. Leiros

Research Group for Host-Microbe Interactions, Department of Medical Biology, Faculty of Health Sciences, University of Tromsø, Tromsø, Norway; Reference Centre for Detection of Antimicrobial Resistance, Department of Microbiology and Infection Control, University Hospital of North Norway, Tromsø, Norway; School of Cellular and Molecular Medicine, Medical Sciences Building, University of Bristol, University Walk, Bristol, United Kingdom; Section of Medical Microbiology III, School of Medical Sciences, Cardiff University, Heath Park Hospital, Cardiff, United Kingdom; The Norwegian Structural Biology Centre (NorStruct), Department of Chemistry, University of Tromsø, Tromsø, Norway.

Metallo-β-lactamas (MBLs) have rapidly disseminated worldwide among clinically important Gram-negative bacteria and have challenged the therapeutic use of β-lactam antibiotics, particularly carbapenems. The *bla* _GIM-1_ gene, encoding one such enzyme, was first discovered in a *Pseudomonas aeruginosa* isolate from 2002 and has more recently been reported in *Enterobacteriaceae*. Here, we present crystal structures of GIM-1 in the apo-zinc (metal-free), mono-zinc (where Cys221 was found to be oxidized), and di-zinc forms, providing nine independently refined views of the enzyme. GIM-1 is distinguished from related MBLs in possessing a narrower active-site groove defined by aromatic side chains (Trp228 and Tyr233) at positions normally occupied by hydrophilic residues in other MBLs. Our structures reveal considerable flexibility in two loops (loop 1, residues 60 to 66; loop 2, residues 223 to 242) adjacent to the active site, with open and closed conformations defined by alternative hydrogen-bonding patterns involving Trp228. We suggest that this capacity for rearrangement permits GIM-1 to hydrolyze a wide range of β-lactams in spite of possessing a more constrained active site. Our results highlight the structural diversity within the MBL enzyme family.

**β-Lactams** have been the most important group of antibiotics used to treat infections (1, 2). However, the dissemination of β-lactams, hydrolytic enzymes that inactivate β-lactams through cleavage of the amide bond of the β-lactam ring, is threatening their clinical longevity (3–5). Production of β-lactamasmes has become the most common β-lactam resistance mechanism in bacteria, particularly in opportunistic Gram-negative species. Metallo-β-lactamasmes (MBLs) hydrolyze all classes of β-lactams except monobactams and are not affected by clinically available β-lactamase inhibitors. Acquired MBL genes are often located on mobile genetic elements (plasmids and transposons) along with other antibiotic resistance genes, potentially resulting in the spread of multidrug resistance. Acquired MBLs reported so far include IMPs, VIMs, NDMs, SPM-1, SIM-1, KHM-1, GIM-1, DIM-1, TMB-1, KHM-1, SMB-1, FIM-1 and AIM-1 (4, 6–9).

The *bla* _GIM-1_ gene was first identified on a 24-kb nontransferable plasmid in 2002 in clinical *Pseudomonas aeruginosa* isolates from Dusseldorf, Germany (10). *bla* _GIM-1_ is embedded in a class 1 integron as a gene cassette and often is accompanied by various aminoglycoside resistance genes. Recent data show that *bla* _GIM-1_ has now moved into *Enterobacteriaceae* (11), and it has also been reported in different clones of multidrug-resistant *P. aeruginosa* isolates from Germany (12). The reported spread into *Serratia marcescens* indicates a potential transmission of mobile genetic elements carrying *bla* _GIM-1_ between different Gram-negative species. Within the accepted MBL classification scheme, GIM-1 belongs to subclass B1 and most closely resembles the IMP enzymes (39 to 43% sequence identity) while showing significant relatedness to the VIM (28% to 31% identity), NDM (28 to 29% identity), and SPM-1 (28% identity) enzymes. The GIM-1 sequence has some unusual features, within the MBL _H_118<sub>x</sub> _H_119<sub>x</sub> _D_120 motif (where _x_ is any residue), containing residues Ser117 and Glu119 that are not frequently found at these positions in other B1 MBLs (10) (Fig. 1).

Kinetic characterization of GIM-1 revealed a broad activity profile with no clear preference between penicillin, cephalosporin, and carbapenem substrates. Compared to IMP-1, VIM-2, and SPM-1, GIM-1 has reduced affinity (K_m) for imipenem, cefoxitin, and ceftazidime (10). GIM-1 is also unusual among the MBLs in hydrolyzing meropenem more efficiently than imipenem (10).

**MATERIALS AND METHODS**

**Cloning, expression, and purification.** The *bla* _GIM-1_ open reading frame (GenBank accession no. Q704V1) beginning at Gln19 (Gln37 according to class B β-lactamase [BBBL] numbering [13]), with additional nucleotides encoding an N-terminal hexa-His tag and tobacco etch virus (TEV) cleavage site, was cloned into the expression vector pDEST14 (Gateway Technology, Invitrogen). The following primers (Sigma-Genosys) were used for amplification of the gene: forward GIM-1 Gln (5’-CAT CAT CAC CAT CAC GAA AAC CTG TAT TTC CAG GGA GCA CAG GGT CAT AAA CCG CTA GAA GGA GAT AGA ACC ATG CGC TTC AGC GG-3’), reverse GIM-1 _attB1_ (5’-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TTA ATC AGC CGC TTC AGC GGG), and forward _attB2-His_ (5’-GGG GAC CAC TTT GTA CAA GAA AGC TGG TGC TTA ATC AGC CGC TTC AGC GGG), and forward _attB2-His_ (5’-GGG GAC CAC TTT GTA CAA GAA AGC TGG TGC TTA ATC AGC CGC TTC AGC GGG). The *bla* _GIM-1_ gene was first amplified using forward GIM-1 Gln and reverse GIM-1 _attB2_ primers, followed by...
another amplification using forward attB1-His and reverse GIM-1 attB2.

The resulting PCR product was used in the BP recombination reaction (to

generate an entry clone) with the pDONR221 vector (Invitrogen Life

Technologies, Great Britain) followed by transformation into Escherichia
coli DH5α (Invitrogen). Sequencing (BigDye Terminator v3.1; Applied

Biosystems) was performed to confirm recovery of the correct clone. The

entry clone was transferred to the pDEST14 vector by the LR recombin-
a tion reaction (to generate an expression clone) and transformed into in-
house-modified E. coli BL21 Star (DE3) pLysS (Invitrogen) cells that also

included the pRARE plasmid (Novagen) for expression and purification.

Expression was induced with 0.5 mM isopropyl-D-thiogalactopyrano-
side (IPTG) for 20 h at 20°C in Luria broth (LB) medium containing 100
µg/ml of ampicillin and 34 µg/ml of chloramphenicol. The soluble lysate
after sonication was purified on a 1-ml His-Trap HP column in buffer A
(50 mM HEPES, pH 7.2, and 100 mM ZnCl₂), washed with 5% buffer B,
and eluted with 5 to 100% buffer B (50 mM HEPES, pH 7.2, 100 mM
ZnCl₂, and 500 mM imidazole). About 6 mg of in-house-made His-

tagged TEV protease was added to GIM-1-containing fractions, and the

mixture was dialyzed overnight at 4°C (10-kDa molecular-size-cutoff
SnakeSkin [Pierce]) with stirring against 10 mM HEPES, pH 7.2, 100 mM
NaCl, 1 mM 2-mercaptoethanol, 1 mM EDTA. A new His-Trap step
removed uncleaved protein and the TEV protease. One pure GIM-1 fraction
was run on an analytical gel filtration column (Superdex 75 26/600) in
buffer C (50 mM HEPES, pH 7.2, 100 mM ZnCl₂, and 150 mM NaCl),
where it eluted as a single peak corresponding to a molecular mass of
approximately 25 kDa.

Crystallization, data collection, and structure determination.

GIM-1 was crystallized by the hanging-drop method, mixing 1 µl protein
(10 mg/ml) with 1 µl reservoir solution (0.1 M Tris, pH 7.1, 21% poly-
ethylene glycol monomethylether 2000 [PEG MME 2K], 4% glycerol).
Crystals grew within 2 days at 22°C. All crystals were cryoprotected with
20 to 25% glycerol, 21% PEG MME 2K, 0.1 M Tris (pH 7.1), and flash
frozen in liquid nitrogen. GIM-1-Apo and GIM-1-Ox were obtained after
soaking the crystals in the cryoprotectant for about 1.5 h with a pyridine
inhibitor. X-ray data collection was performed at the European Synchro-
tron Radiation Facility (ESRF), France, at beam line ID29. The images
were integrated and scaled with XDS (14), and structure factors were obtained using TRUNCATE (15). The phase problem was solved by PHASER (16) using IMP-1 (Protein Data Bank [PDB] entry 1DDK) as a search model (17), and the structures were refined in Phenix (18) and manually built in WinCoot (19).

**Protein structure accession numbers.** Coordinates and structure factors of GIM-1, GIM-1-2Mol, GIM-1-Apo, and GIM-1-Ox have been deposited in the Protein Data Bank under accession numbers 2YNT, 2YNW, 2YNU, and 2YNV, respectively.

**RESULTS AND DISCUSSION**

Here, we report three-dimensional structures of native GIM-1 in two crystal forms (GIM-1 and GIM-1-2Mol), along with the metal-free apo-zinc structure (GIM-1-Apo) and a structure with the zinc ligand Cys221 oxidized in chain A and only one of the two zinc ions present for chains A and B (GIM-1-Ox). The resolutions obtained were 1.60, 1.70, 2.06, and 2.05 Å, respectively (Table 1). All structures were obtained from similar crystallization conditions (Table 1), and no reducing agents were added. The apo- and mono-zinc (oxidized) structures were derived from unsuccessful soaks with candidate inhibitors (pyridines with a carboxyl group(s)).

The GIM-1 structure has the typical MBL αββαβα fold with two Zn2+ ions situated in the groove between the two core β-sheets. The overall GIM-1 structure has an RMSD for CA atoms of 1.01 Å compared to IMP-1 (PDB 1DDK; 44% sequence identity), 1.40 Å compared to VIM-2 (PDB 1KO3; 35% sequence identity), and 1.38 Å compared to VIM-7 (PDB 2Y8B; 36% sequence identity). The native active sites (GIM-1 and GIM-1-2Mol) contain two zinc ions (Zn1, a trihistidine site, and Zn2, a Cys-His-Asp site) bridged by a water molecule and separated by a distance of 3.45 to 3.50 Å, where Zn1 displays a tetrahedral coordination and Zn2 seems to be in a distorted trigonal bipyramidal coordination with one vacant position (Fig. 3a and b) (see Table S1 in the supplemental material). This contrasts with the majority of B1 MBLs where tetrahedral geometry is generally observed for the Zn1 site, while Zn2 often has an additional apical water as a fifth ligand, resulting in a trigonal bipyramidal coordination. In the GIM-1-Apo structure, the electron density indicates an absence of zinc, with the two Zn1 ligands His116 and His118 rotated relative to their positions in the native structures, increasing the space between the three Zn1 His ligands (Fig. 3c). In the oxidized form (GIM-1-Ox) Zn2 is absent, and only Zn1 is present in both chains A and B. However, electron density maps provide strong evidence for oxidation of Cys221 in the A chain only (Osc221) (Fig. 3d). We surmise that the unsuccessful inhibitor soak that preceded collection of the GIM-1-Ox data set resulted in the loss of Zn2, suggesting that the GIM-1 Zn2 site is of lower affinity than the Zn1 site. In other MBLs, the low Zn2 preference has been related to the presence of subclass B1 MBLs (BcII, SPM-1, VIM-7, and VIM-2 [20–23]).

In subclass B1 MBLs such as GIM-1, the active site is flanked by two extended loops, loop 1 and loop 2 (Fig. 2a), that connect elements of secondary structure. Of these, the flapping loop (loop 1; residues 60 to 66) is proposed to act as a flexible lid that closes...
FIG 2 (a) Superposition of the GIM-1 structures showing the Zn$^{2+}$ ions and Zn$^{2+}$ ligands with GIM-1 chains A (cyan), B (dark blue), and C (light blue); GIM-1-2Mol chains A (black) and B (magenta); GIM-1-Apo chains A (yellow) and B (tan); and GIM-1-Ox chains A (gray) and B (green). (b) Interactions involving Trp228 in the closed GIM-1 chain A (cyan) and open GIM-1-2Mol chain A (gray). Calculated electrostatic surfaces of superimposed closed GIM-1 (chain A) (c), open GIM-1-2Mol (chain A) (d), IMP-1 (PDB 1DD6) (e), and VIM-7 (PDB 2Y87) (f), where ceftazidime is docked in VIM-7 (22), are also shown. All figures were made with PyMol (http://www.pymol.org).
over the active site to promote binding and hydrolysis of substrates. In the various GIM-1 structures this loop adopts a range of conformations, being most open in the native GIM-1-2Mol (chains A and B) structure and most closed in the GIM-1 (chain A) structure, with a maximal difference in the main-chain positions of the two conformations of 7 Å for Glu62 (Fig. 2b). Residues in loop 1 implicated in binding of substrates/ligands to other B1 MBLs include Phe61 and Tyr67 in VIM-2 (20), Val61 and Trp64 in IMP-1 (17), and Leu61 and Tyr67 in VIM-7 (22) (Fig. 2e and f). The presence of isoleucine, tyrosine, and valine at residues 61, 64, and 67, respectively, and the conformational variability of this region in the different structures, are consistent with GIM-1 loop 1 adopting a similar role.

Loop 2 in B1 MBLs (also called L10) comprises residues 223 to 242 and faces loop 1 across the active-site groove. In this region, GIM-1 is distinguished from other B1 MBLs by the presence of arginine at position 224, tryptophan at 228, and tyrosine at 233 (Fig. 1). The presence of isoleucine, tyrosine, and valine at residues 61, 64, and 67, respectively, and the conformational variability of this region in the different structures, are consistent with GIM-1 loop 1 adopting a similar role.

In all, loop 1 implicated in binding of substrates/ligands to other B1 MBLs include Phe61 and Tyr67 in VIM-2 (20), Val61 and Trp64 in IMP-1 (17), and Leu61 and Tyr67 in VIM-7 (22) (Fig. 2e and f). The presence of isoleucine, tyrosine, and valine at residues 61, 64, and 67, respectively, and the conformational variability of this region in the different structures, are consistent with GIM-1 loop 1 adopting a similar role.

Loop 2 in B1 MBLs (also called L10) comprises residues 223 to 242 and faces loop 1 across the active-site groove. In this region, GIM-1 is distinguished from other B1 MBLs by the presence of arginine at position 224, tryptophan at 228, and tyrosine at 233 (Fig. 1). In eight of the nine GIM-1 models, the Arg224 side chain makes hydrog bonds, with one or both of the NE and NH1 atoms, to Gly230 O and creates a positively charged R2 binding site (Fig. 2c and d), i.e., the binding site for the C2/C3 substituents of carbapenem or cephalosporin substrates. However, this does not seem to be discriminatory against substrates (cephalosporins) with positively charged C3 substituents, since GIM-1 binds ceftazidime relatively tightly (K_m of 31 μM) but cefepime more weakly (K_m of 431 μM) (10). GIM-1 is unique among B1 MBLs of known sequence in possessing tryptophan at position 228. Compared to other B1 MBLs of known structure (e.g., VIM-2, VIM-7, IMP-1, and NDM-1), the side chain of GIM-1 Trp228 both limits the size of the active-site groove in the R2 pocket and participates in hydrogen-bonding interactions that vary between the different structures. In the GIM-1-2Mol chain A structure, Trp228 NE1 is hydrogen bonded to the main-chain carbonyl of the Zn2 ligand His263, while in chain A of the GIM structure it instead contacts OD1 of Asp68 at the base of loop 1 (Fig. 2b). The bond of Trp228 NE1 to His263 O is found in five of the nine GIM-1 models. In the GIM-1-Apo structure, Trp228 is partly disordered in chains A and B, so there could be

**FIG 3** 2Fo-Fc electron density maps of GIM-1 (Mol A at 1.5σ) (a), GIM-1-2Mol (Mol A at 1.5σ) (b), GIM-1-Apo (Mol A at 1.1σ) (c), and GIM-1-Ox (Mol A at 1.5σ) (d). For all panels, Fo-Fc difference maps are displayed at 4.0σ (green) and ~ 4.0σ (red).
an interplay between Trp228 and the metals. In other B1 MBL structures (e.g., VIM-2, VIM-7, IMP-1, and NDM-1), an interaction from the His263 main-chain oxygen atom with a loop 2 residue is not featured. For other apo MBL structures, e.g., NDM-1 (PDB 3SPU) or L1 (PDB 2FU6), the effects of removing the metal ions are primarily reported in loop 1 and loop 2. However, for GIM-1-Apo, the main effect seems to be for residues 232 to 237 in loop 2, which are disordered (chain B) or have elevated B-factors (chain A). Loop 1 has conformations which are variable, from the most open conformation (GIM-1-2Mol chain A and B) to the most closed conformation (GIM-1 chain A) (Fig. 2c and d) among the GIM-1 structures which both contain two zinc ions, suggesting that the metal does not seem to be determining the loop 1 conformation. Our structures are suggestive of considerable plasticity in this region of the active-site groove, which is consistent with the ability of GIM-1 (like other B1 MBLs) to bind and hydrolyze a wide variety of differently substituted substrates. The presented GIM-1 structures imply that both loop 1 and loop 2 can adapt their conformations during catalysis. Other studies on MBLs have shown a complex interplay between sequence and structure, revealing that loop flexibility, protein stability, and particular mutations are important for biological function and efficiency (24–26). Some of the described effects are also ligand dependent.

Residue 233, normally asparagine in B1 MBLs, has been studied in several such enzymes, but its role(s) in substrate binding and/or as a contributor to a putative oxanion hole remain unclear and controversial (24–27). In GIM-1, tyrosine is found at position 233 and makes water-mediated hydrogen bonds from the O and N main-chain atoms to His196 and the side-chain hydroxyl to Asp236. We suggest that the Tyr233 side chain provides a hydrophobic surface for interactions with bound substrates through hydrogen bonds, as would be required for a role in an oxanion hole. Asn233 is conserved in ~67% (26) of all class B1 MBL sequences, and its alteration, as in a recent study on the IMP-1 enzyme, where 19 mutants of Asn233 were investigated, can significantly affect kinetic parameters (26). However, while expression of the IMP-1 Asn233Tyr mutant reduced the M. cefotaxime compared to that for the wild-type enzyme, values for ampicillin, imipenem, or cephalothin were unaffected (26). These data, together with the presence of Tyr233 in B1 MBLs such as BlaB (28) and IMP-14 (GenBank accession no. AY553332), indicate that substitution of tyrosine for asparagine at position 233 is unlikely to have significant consequences for the binding and hydrolysis of substrates by GIM-1. However, our structures suggest that the bulky tyrosine side chain at this position in the GIM-1 structures serves to limit the size of the drug binding pocket and possibly constrain the mobility of loop 2 (Fig. 2c and d).

Superposition of the various GIM-1 structures upon those for other B1 MBLs indicates that compared to, e.g., the IMP or VIM enzymes, the GIM-1 active-site groove is more confined (due to the presence of Ile61 in loop 1 and, in particular, to Trp228 and Tyr233 in loop 2) and is also positively charged (Fig. 2). The area and volume of the antibiotic binding, where cephalazin is docked into VIM-7 (22), is smaller for GIM-1 (GIM-1 chains A and B, GIM-1-2Mol chains A and B, and GIM-1-Ox chain A) than for IMP-1 (PDB 1DD6) and VIM-7 (PDB 2Y87) using the CASTp server (29). The availability of multiple structures from this study also highlights the mobility of both loops 1 and 2, suggesting that, despite the narrower active-site groove of GIM-1, a wide variety of substrates still can be accommodated by reorientation of these regions (that may in particular be associated with alternative hydrogen bonding by the Trp228 side chain). This is consistent with the relatively low $K_m$ values obtained for hydrolysis of a broad range of $\beta$-lactams by GIM-1. Notably, however, GIM-1 binds relatively weakly (as inferred from elevated $K_m$ values) to $\alpha$-methoxy-substituted substrates, such as cefoxitin and moxalactam, that are often reasonably good substrates for related enzymes. This indicates a more limited ability of the restricted GIM-1 active site to accommodate this type of substituent.

In conclusion, our study demonstrates that the GIM-1 MBL contains three features that are typical of B1 MBLs (a binuclear zinc site in an $\alpha/\beta/\beta$ fold, mobile loops 1 and 2 adjacent to the active site, and a lower-affinity Zn2 site) and some unique characteristics, with the presence of Trp228 and Tyr233 creating a narrower and more hydrophobic active-site groove. In particular, the two distinctive hydrogen binding networks involving Trp228 (interacting with either His263 or Asp68) seem to be a novel feature of the GIM-1 enzyme. The availability of nine independently refined models of the GIM-1 MBL provide a more dynamic picture of the active site than is normally provided by crystallographic investigations, highlighting the plasticity of the active-site groove that is a feature of these enzymes. The high-resolution structural information provided here will facilitate the inclusion of GIM-1 in structure-based studies aimed at developing inhibitors that are effective against the range of mobile MBLs and thus aid our efforts to counter the growing threat of MBL-mediated antibiotic resistance.

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REFERENCES

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Supplementary material:

**Crystal structures of Pseudomonas aeruginosa GIM-1: active site plasticity in metallo-β-lactamases**

Pardha Saradhi Borra, Ørjan Samuelsen, James Spencer, Timothy R. Walsh, Marit Sjo Lorentzen and Hanna-Kirsti S. Leiros

**Table S1.** a) Inter atomic distances (Å) and b) angles for the ligand-Zn-ligand in the active site of GIM-1, GIM-1-2Mol and GIM-1-Ox structures.

### a)

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### b)

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*The Zn2 and W1 atoms are absent in GIM-1-Ox