Kinetic and Structural Characterization of the First B3 Metallo-β-Lactamase with an Active Site Glutamic Acid

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Abstract

The structural diversity in metallo-β-lactamases (MBLs), especially in the vicinity of the active site, has been a major hurdle in the development of clinically effective inhibitors. Representatives from three variants of the B3 MBL subclass, containing either the canonical HHH/DHH active site motif (present in the majority of MBLs in this subclass) or the QHH/DHH (B3-Q) or HRH/DQK (B3-RQK) variations were reported previously. Here, we describe the structure and kinetic properties of the first example (SIE-1) of a fourth variant containing the EHH/DHH active site motif (B3-E). SIE-1 was identified in the hexachlorocyclohexane-degrading bacterium *Sphingobium indicum*, and kinetic analyses demonstrate that although it is active against a wide range of antibiotics its efficiency is lower than that of other B3 MBLs, but with improved efficiency towards cephalosporins relative to other β-lactam substrates. The overall fold of SIE-1 is characteristic of the MBLs; the notable variation is observed in the Zn1 site due to the replacement of the canonical His116

by a glutamate. The unusual preference of SIE-1 for cephalosporins and its occurrence in a widespread environmental organism suggests scope for increased MBL-mediated β-lactam resistance. It is thus relevant to include SIE-1 into MBL inhibitor design studies to widen the therapeutic scope of much needed anti-resistance drugs.

Introduction

With respect to microbial infections, antibiotic resistance is a primal threat to the application of modern medicine. Our understanding of the prevention of infection through sanitation, hygiene and nutrition has increased significantly in the last century (1). Despite this, the accelerated rise of antibiotic resistance endangers our ability to treat even simple infections, which globally increases the death toll associated with microbial infections, particularly in immunocompromised patients (*e.g.* those undergoing invasive surgery, organ transplants and/or chemotherapy) and in underdeveloped countries, but also among the elderly population in industrialised nations (1, 2).

β-Lactams are the most commonly used antibiotics, a group of drugs that consists of four main classes, penicillins, carbapenems, cephalosporins and monobactams (3). All of these classes utilize the four-membered β-lactam ring to inhibit transpeptidases involved in bacterial cell wall synthesis (3, 4). The most common strategy used by Gram-negative bacteria to deactivate these compounds is hydrolysis of the β-lactam ring system, a reaction catalyzed by a group of enzymes termed β-lactamases (4). β-Lactamases can be organized into two main groups, serine-β-lactamases (SBLs, Ambler Class A, C and D) and metallo-β-lactamases (MBLs, Ambler Class B) (5). SBLs utilize an active site serine residue as a nucleophile during hydrolysis of the β-lactam ring, whereas MBLs facilitate hydrolysis through a hydroxide nucleophile bound to either one or two Zn^{2+} ions (6-9). MBLs can be further classified into one of three subclasses (B1, B2 or B3) based on their active site structure, the number of Zn^{2+} ions required for maximum activity and their substrate profile (6, 7, 9, 10). The active sites of MBLs contain two (closely spaced) metal binding sites (Zn1 and Zn2), which are formed by six distinct amino acid side chains (three in each site); the identity of these side chains varies between subclasses (11).

B1 MBLs are characterized by the canonical HHH/DCH active site motif for the Zn1/Zn2 sites, respectively, and include numerous variants of the enzymes NDM, VIM and IMP. B1 MBLs are the clinically most relevant and widespread MBLs, and are commonly located on mobile genetic elements (6-9). B2 MBLs feature the NHH/DCH active site motif, are less common than B1 MBLs, display a distinct preference for carbapenem antibiotics and are active with a single Zn^{2+} present in

the active site (6-9). They are largely chromosomally encoded, which limits their distribution via horizontal gene transfer significantly (9). Both B1 and B2 MBLs have emerged from a common ancestral gene (9, 12). In contrast, the B3 subgclass is phylogenetically distinct from other MBLs and shares less than 20% sequence similarity with B1 and B2 MBLs (3, 11).

In contrast to B1 and B2 MBLs the B3 subclass possesses greater diversity in its active site composition. A recent sequence analysis indicates the presence of four B3 MBL active site variations (see Table 1) (11). The canonical and most abundant variant is characterised by the HHH/DHH active site motif. The second most abundant representative displays a variation in position 116 from histidine to glutamine in the Zn1 site (**Q**HH/DHH motif; variation shown in bold). To date, one representative from this B3-Q MBL group has been characterised (the enzyme GOB) and its structure and catalytic properties are similar to those of the canonical B3 MBLs (13, 14). The rarest of the four variants carries three active site variations: one in the Zn1 site (His118 to arginine) and two in the Zn2 site (His121 to glutamine and His263 to lysine), resulting in the unusual H**R**H/D**QK** active site motif. Representatives from this group have been found to be sensitive to inhibition by clavulanic acid, a clinical inhibitor of many SBLs, which has virtually no effect on known MBLs (11). This change in kinetic behavior has been attributed to the variation found in position 263, decreasing the metal affinity in the Zn2 site (11). The fourth B3 MBL variant displays the **E**HH/DHH active site motif, where His116 in the Zn1 site is replaced by a glutamate residue. However, no representative from this B3-E group has yet been characterized.

It may be anticipated that this relatively conserved substitution in the Zn1 site will not lead to major changes of the structure, function and catalytic efficiency of SIE-1 when compared to canonical B3-MBLs. However, gaining insight into the properties of naturally occurring variants of MBLs may enable new strategies to develop MBL inhibitors as clinically useful treatments to combat the further spread of antibiotic resistance. The identification of the clinical relevance of position 263 in the Zn2 active site of B3-RQK MBLs is an example supporting this hypothesis (11).

While MBLs from the B1 subclass are currently the most established and clinically relevant among this family of enzymes, MBLs from the B3 subclass have emerged as an alternative cause of concern. There appears to be a large reservoir of B3-type

MBLs in environmental microorganisms, *i.e.* organisms that are not considered as human pathogens but that dwell in diverse environments that are not yet impacted by human activities. Such organisms and their B3 MBLs thus represent a possible reservoir for future enzyme activities that contribute to antibiotic resistance (11, 15-21). Indeed there is evidence to suggest that B3 MBLs from environmental sources have, on multiple occasions, carried through to clinical settings (11). This is supported by the phylogenetic diversity of the characterized B3 MBLs which have been isolated from clinical samples. As such it is important to monitor these environmental sources in order to design inhibitors and antibiotics which are effective against all available MBL variants (22-26).

We selected the B3-E MBL identified in the genome of the hexachlorocyclohexane-degrading bacterium *Sphingobium indicum* as a target for enzymatic characterization henceforth referred to as SIE-1 (derived from ***S****phingobium* ***i****ndicum* B3-**E** MBL). *Sphingomonadaceae* is a family of aerobic chemoheterotrophs which are widespread throughout environmental and human related settings (*e.g.* corals, soils, plant surfaces, drinking water and haemodialysis fluids) as well as in clinical samples (27-30). These bacteria also have a remarkable resilience towards harmful chemicals such as hexachlorocyclohexane (27, 31). This resilience along with their environmental abundance raises concerns about their potential to harbor and disseminate resistance genes (28, 32-34).

Materials and Methods

Materials

The pET-24a(+) plasmid containing the SIE-1 gene was purchased from Gene Universal Inc. All purification equipment (both chromatography columns and fast protein liquid chromatography systems) was purchased from GE Healthcare. All chemicals and buffers were purchased from the Sigma Chemical Company. SnakeSkin™ dialysis tubing was purchased from Thermo Fisher Scientific. All kinetic assays were run using a Cary 60 Agilent UV-Vis spectrophotometer with a Varian Single Cell Peltier Accessory for temperature control.

Expression and Purification

The open reading frame containing SIE-1 was cloned into pET-24a(+). The vector was transferred into *Escherichia coli* BL21 (DE3) cells via heat shock. The transformed cells were grown in LB medium containing 100 µg/mL kanamycin for selection and 0.2% lactose for induction of protein expression. The cells were initially grown at 37 ^{o}C and 180 rpm until they reached an OD_{600} of ~0.5, after which the temperature was reduced to 18 ^{o}C and the incubation continued overnight. The cells were then harvested by centrifugation and chemically lysed by resuspension in 50 mM Tris buffer, pH 8.0, containing 0.3 M NaCl, 1% TritonX-100, 1 M urea, 1 mg/mL lysozyme, 1 mg/mL DNase I and 1.5 mg/mL EDTA-free protease inhibitor cocktail. The resuspended cells were stirred at 4 ^{o}C for 20 minutes and then for another 30 minutes at room temperature. Subsequently, the lysate was centrifuged at 14,000xg for 40 minutes and the supernatant was transferred into SnakeSkin dialysis tubing. Dialysis was performed with three changes of 2 L of 25 mM Tris buffer, pH 8.0, containing 0.1 mM $ZnCl_2$; the incubation period between buffer changes was 3 hours of stirring at 4 ^{o}C. Following dialysis, the protein was loaded onto a HiPrep SP FF 16/10 cation exchange column and purified using a 20 column volume (CV)-gradient from 0 - 0.5 M NaCl. Fractions containing SIE-1 (determined from SDS-PAGE analysis) were pooled and 1 M NaCl was added before the solution was loaded onto a HiLoad 26/10 Phenyl Sepharose HP reverse phase column. The enzyme was then eluted using a 10 CV gradient from 1 – 0 M NaCl. Fractions containing SIE-1 were again pooled. Excess NaCl was removed *via* buffer exchange using EconoPac 10DG desalting columns (Bio-Rad). The purified enzyme was analyzed by SDS-

PAGE analysis and size-exclusion chromatography with multi-angle light scattering (SEC MALS). The sample was at least 95% pure with a molecular weight of ~35 kDa, in agreement with the weight calculated from the amino acid sequence. The purified protein was stored at 4 ^{o}C in 20 mM Tris buffer, pH 8.5.

Kinetic Assays

The β-lactamase activity of SIE-1 was tested against a range of β-lactam antibiotics. Activity was measured by monitoring the degradation of meropenem (λ = 297nm, ε = 6500 $M^{-1}cm^{-1}$), imipenem (λ = 295nm, ε = 9000 $M^{-1}cm^{-1}$), biapenem (λ = 293nm, ε = 8630 $M^{-1}cm^{-1}$), cephalothin (λ = 265nm, ε = 8790 $M^{-1}cm^{-1}$), cefuroxime (λ = 260nm, ε = 9320 $M^{-1}cm^{-1}$), nitrocefin (λ = 485nm, ε = 17400 $M^{-1}cm^{-1}$), carbenicillin (λ = 232nm, ε = 1190 $M^{-1}cm^{-1}$), penicillin G (λ = 235nm, ε = 936 $M^{-1}cm^{-1}$), and ampicillin (λ = 235nm, ε = 900 $M^{-1}cm^{-1}$) over 1 minute at 25^{o}C. Assays were run in 10 mM Tris buffer, pH 8.5, with 100 μM Zn^{2+} in the enzyme stock solution. Assays were run in 10 mM Tris buffer, pH 8.5, with 100 μM Zn^{2+} in the enzyme stock solution. Note that the enzyme was separately expressed, purified, and assayed in the absence of added Zn^{2+} without measurable effect on the catalytic properties of SIE-1 suggesting that sufficient amounts of zinc are present in the culture medium to promote full enzyme activity (data not shown).

Inhibition assays were run under the same conditions using meropenem as the substrate. Activity measurements were carried out in the presence of increasing concentrations of the known MBL inhibitor captopril (0 μM, 5 μM, 10 μM, and 20 μM) (35) using an enzyme concentration of 3 nM and substrate concentrations ranging from 25 – 250 μM. Inhibition data were fit using the mixed inhibition model equation (35).

Crystallographic Analysis

The SIE-1 solution was concentrated to 13 mg/mL. Crystallization trials were performed using hanging drop vapor diffusion at 20 ^{o}C. Drops were made using 200 nL of the 13 mg/mL enzyme solution mixed with 200 nL of well solution. SIE-1 crystallized in 0.2 M $LiSO_4$, 0.1 M Tris buffer, pH 8.5, and 1.26 M NH_4SO_4. Protein crystals were collected and cryoprotected by adding 20% glycerol to the well solution.

X-ray data were collected remotely on the MX-1 beamline at the Australian Synchrotron (Melbourne) and were processed with XDS (36, 37). Model refinement and building was conducted using PHENIX 1.15.2 and COOT 0.8.9.2, respectively (38, 39). Initial phasing was performed using the crystal structure of the canonical B3 MBL SMB-1 (accession number 3VPE) (40).

The final model and structure factors were submitted to the Protein Data Bank under the accession number 7LUU. Enzyme-ligand interactions were visualized and figures were prepared using Mol* and PyMOL, respectively (41, 42). Crystallographic and refinement data are summarized in Table 2.

Results and Discussion

SIE-1 is a potent MBL with a preference for cephalosporin substrates

In an *in vivo* assay SIE-1 has been previously shown to confer resistance to *E. coli* against a range of substrates representing each of the major clinically applied β-lactam groups (*i.e.* penicillins, cephalosporins and the "last-resort" carbapenems) (11). Here, we probed the *in vitro* catalytic efficiency of SIE-1 by recombinant expression in *E. coli* and purification to homogeneity *via* sequential ion exchange and reverse phase chromatography. Apparent catalytic parameters (k_{cat}, K_M) are summarized in Table 3 and compared with corresponding values from representative enzymes from each of the B3 MBL active site variants. In contrast to B3 MBLs with the canonical HHH/DHH active site motif or its QHH/DHH variant (*e.g.* L1, AIM-1 and GOB-1), SIE-1 is not very efficient in hydrolyzing penicillin G and the related ampicillin (13, 14, 43-49). The loss of efficiency is largely due to its modest binding affinity for these substrates, with high K_M values ranging from 450 μM to 2 mM. Although still less efficient than its canonical and B3-Q counterparts, SIE-1 is reactive towards carbapenem-type substrates (*i.e.* meropenem and imipenem), largely due to significantly enhanced binding interactions when compared to penicillin G or ampicillin. SIE-1 has a catalytic efficiency for the inactivation of cephalosporins that is superior to that measured for penicillin derivatives or carbapenems, which is unusual among B3 MBLs. Overall, the *in vitro* kinetic assays demonstrate that the B3-E representative is slightly less reactive than its canonical and B3-Q counterparts, but is comparable to the B1 MBL NDM-1, an MBL that has caused grave clinical concerns (Table 3).[*]

MBLs pose a major concern to health care because, to date, no clinically useful inhibitors for these enzymes are available (22, 24-26, 50-53). Compounds such as captopril are potent MBL inhibitors (11) but cannot be used to combat antibiotic resistance since they are either not specific enough (*i.e.* they bind to other metallohydrolases) or may be used as treatments for other ailments. Captopril, for instance, inhibits the angiotensin-converting enzyme (ACE) and as such is used for

[*] While there is agreement between the *in vitro* catalytic activity and the *ex vivo* resistance conferred by SIE-1 for the majority of antibiotics tested, for two of them (meropenem and cephalothin) that is not the case. A similar observation was also made for AIM-1 (11). It is possible that in the disc diffusion tests used in these *ex vivo* assays the amount of these antibiotics exceeded their minimum inhibitory concentrations (MICs). Indeed, we performed an E-Test strip analysis with meropenem to demonstrate that the recombinant expression of SIE-1 increases the MIC of *E. coli* cells from 0.064 μg/mL to 0.19 μg/mL.

the treatment of hypertension and some types of congestive heart failure (54). Furthermore, compounds such as clavulanic acid or avibactam are efficient inhibitors of some (Class A) SBLs (and hence are often present in β-lactam antibiotic prescriptions) but they have proven ineffective against MBLs (11). A notable exception are the MBLs from the B3-RQK subclass (11, 55). Therefore, the effects of captopril, clavulanic acid and avibactam on the catalytic properties of SIE-1 was investigated.

The presence of clavulanic acid or avibactam had no impact on the catalytic activity of SIE-1 (data not shown). However, significant inhibition was observed in the presence of captopril (Figure 1). A simultaneous fit of the data to an equation describing inhibition (11) supports a mixed-type of inhibition with a competitive and uncompetitive component, characterized by a K_{ic} of 9 ± 2 μM and a K_{iuc} of 23 ± 12 μM. The magnitude of the two inhibition constants is well within the recorded inhibitory effects of captopril on MBLs (18). The predominantly competitive mode of inhibition is consistent with that of previously studied MBLs (including structural analyses of captopril-B1 MBL complexes (18)), but the significant contribution from an uncompetitive mode of binding indicates that captopril has some flexibility in its interactions with SIE-1 (43, 56, 57). This mixed mode inhibition by captopril has been previously observed with another MBL from the canonical B3 subclass, AIM-1 (43). Structural data that illustrate the binding of captopril to B3 MBLs is sparse, with only three inhibitor-bound crystal structures reported to date, all for enzymes with the canonical B3 MBL motif in the active site (*i.e.* L1 (PDB code 2FU8), FEZ-1 (1JT1) and SMB-1 (5AYA)) (56-58). The consensus among these structures indicates that the predominant interaction by which competitive inhibition is facilitated is through the substitution of the bridging hydroxide, *i.e.* the nucleophile that initiates hydrolysis of the β-lactam substrates, with the thiol group of captopril (56-58). A similar observation with respect to the displacement of the bridging hydroxide was made with other inhibitors of B3 MBLs, including mercaptoacetate, 4-nitrobenzenesulfonamide and 2-mercaptoethanesulfonate (40, 56, 59). Apart from the displacement of the metal ion-bridging hydroxide group, captopril is able to interact with a number of amino acid side chains via hydrogen bonds, as well as electrostatic and hydrophobic interactions (23). Since the sequence conservation outside the immediate first coordination sphere among the B3 MBLs is considerably

lower than in the metal ion binding site the influence of these interactions on the inhibition can vary significantly (40). It should be pointed out that B1 MBLs, although not the subject of the current work, are also inhibited by captopril in a largely competitive mode, with inhibition constants of similar magnitude to those reported for B3 MBLs (23, 60). Since the active site composition (including their interactions with the metal ions) in B1 MBLs and canonical B3 MBLs are substantially conserved it seems plausible that the displacement of the metal ion-bridging hydroxide by the thiol group of the inhibitor is the most significant contribution to the binding of captopril to MBLs.

SIE-1 is the first example of a native MBL with a glutamate residue as Zn^{2+} ligand in the active site

From the four active site variants among the B3 MBLs, three have been structurally characterized, including representatives with the canonical HHH/DHH motif (*e.g.* AIM-1), the closely related QHH/DHH motif (*i.e.* GOB-18) and the unusual HRH/DQK motif (*i.e.* CSR-1) (11, 46, 61). No structural data for MBLs from the B3-E subclass have yet been reported. Here, crystals of SIE-1 were obtained and X-ray diffraction data collected to 1.68 Å resolution (Table 2). The final model includes 265 residues (Ser39 to Glu309); the 20 N-terminal and three C-terminal residues are not observed (note the standard BBL numbering scheme is used throughout this report (62)). The N-terminal sequence of SIE-1 consists of 36.8% alanine residues suggesting that this region may not adopt secondary structure features but instead forms a dynamic, flexible tail and hence is not captured in the crystal structure. Overall, the structure is well defined with a low R-factor and R-free value (0.1412 and 0.1700, respectively); 96.97% of the residues are favored in the Ramachandran plot, with no outliers. The overall structure displays the typical MBL fold, forming an αβ/βα sandwich that flanks the binuclear Zn^{2+} active site (Figure 2A). As expected, the only variation in the active site is the presence of a glutamate in position 116 in the Zn1 site, replacing the canonical histidine (Figure 2B). This glutamate side chain coordinates monodentate to the Zn^{2+} ion, resulting in a coordination environment that is very similar to that of canonical B3 MBLs. However, there is an interesting correlation between the metal-metal distance in the active site of B3 MBLs and their catalytic efficiency. For the canonical AIM-1, the B3-Q GOB-18 and the B3-E SIE-1 the Zn^{2+}-

Zn^{2+} distances are 3.48 Å, 3.52 Å and 3.75 Å, respectively, and it thus appears that the shorter metal distance correlates to enhanced reactivity, at least with respect to some substrates (see Table 3). Note that the B3-RQK representative CSR-1 has not been included here as its structure does not have bound metal ions, due to their reduced affinity (11).

In SIE-1 the Zn1 site adopts a tetrahedral geometry using the side chains of Glu116, His118 and His196 as ligands, together with the oxygen from the metal ion-bridging water/hydroxide (Wat1 in Figure 2B). The Zn2 site assumes a distorted octahedral geometry. The six ligands are contributed from the three amino acids of the Zn2 motif (*i.e.* Asp120, His121 and His263), the oxygen of the metal ion-bridging water/hydroxide (Wat1) and a second, terminally coordinated water molecule (Wat2 in Figure 2B). The hydroxyl group of the bound glycerol, added as cryoprotectant to the crystallization mixture, provides the sixth ligand.

Typically, the Zn2 site in free canonical B3 MBLs adopts a five-coordinate environment with distorted square pyramidal or trigonal bipyrimidal geometries. In contrast, the B3-Q representative GOB-18 adopts a tetrahedral Zn2 site (40, 45, 46, 61, 63). However, as demonstrated in the structure of the canonical B3 MBL SMB-1, the binding of a substrate molecule alters the geometry of the Zn2 site to octahedral, with the substrate forming two bonds with that metal ion (Figure 3) (56). Thus, the bound glycerol in combination with Wat2 in the structure of SIE-1 may mimic binding interactions of substrate molecules in the active site. We have been unsuccessful in obtaining SIE-1 crystals in the presence of the inhibitor captopril, possibly a reflection of the two binding modes that are possible (competitive and uncompetitive; see above). *In silico* docking indicated that the preferred mode of binding involves an interaction between the thiol group of the inhibitor and both Zn^{2+} ions in the active site, as observed in the crystal structures of other MBLs (*e.g.* SMB-1 (59)).

In MBLs sequence similarity beyond the active site is generally low. However, a small number of amino acid side chains in the outer coordination sphere appear to be well conserved in a number of B3 MBLs. Residues in positions 41, 221 and 223 have been shown to be involved in substrate binding in almost all B3 MBLs (44, 46, 56, 61, 63).

In position 41 of B3 MBLs a tryptophan is present in representatives from each active site variant, and in position 221 all but the B3-Q GOB-18 have a serine

residue (in the latter the alcohol functional group of serine is replaced by an S-methyl thioether from a methionine residue). In position 223 the degree of variability is larger, but all active site variants except B3-RQK employ polar side chains (asparagine, serine or threonine; alanine in the B3-RQK CSR-1). Furthermore, the residue in position 157 in canonical B3 MBLs (mostly a glutamine, but a histidine in L1) has also been shown to play an important role in substrate binding (56, 61, 63). Similarly, SIE-1 contains a glutamine in this position and GOB-18 has a glutamate, whereas CSR-1 again stands out employing an alanine instead. In SIE-1, Gln157 forms a hydrogen bond (2.77 Å) with one of the terminal (primary) hydroxide groups of the bound glycerol molecule (Figure 4A). This interaction is reminiscent of the active site interactions between the oxygen in the 1-hydroxyethyl group of a hydrolyzed β-lactam substrate and Gln157 in SMB-1 (Figure 4B) (56, 63). Similarly, Ser221 interacts with the other primary hydroxyl group of glycerol, forming a weaker hydrogen bond (2.97 Å). Trp41 and Asn223 are not directly involved in interactions with glycerol in SIE-1 but may play an important role in binding and orienting β-lactam substrates in this enzyme. Considering the high degree of similarity in those four positions associated with substrate binding it is anticipated that SIE-1 has a similar affinity and preference for β-lactams like MBLs from the canonical B3 and B3-Q groups. This argument is at odds with the broadly larger K_m values noted for SIE-1 and its preference for cephalosporins (Table 3). In addition to secondary coordination sphere residues the N-terminal tail has also been shown to have an effect on substrate binding in various MBLs (including the B1 MBL NDM-1) (11, 63). This effect is most clearly seen in the B3-RQK enzyme CSR-1, where the N-terminal tail is positioned such that binding of substrates and even the catalytically essential Zn^{2+} ions to the active site are obstructed (11). Access to the active site is only gained upon the removal of this N-terminal tail, indicating the presence of an as of yet unidentified regulatory mechanism that may activate CSR-1 by either removing or shifting this N-terminal end. While in the canonical B3 MBLs AIM-1 and L1 the N-terminal tail is prevented from obstructing the active site via disulfide bridge formation or oligomerisation, in the canonical B3 enzyme Rm3 the presence of an extended α-helix preceding Trp41 (Figure 5) prevents the N-terminus from bending away from the active site (61, 63, 64). Thus, a deeper active site groove is created, limiting the number of suitable substrates able to bind to the active site (63). In

contrast, in SIE-1 the N-terminal end is not resolved in the crystal structure indicating a degree of flexibility. It is thus likely that residues within this mobile region play an important role in binding and orienting substrates, which may contribute to the observed preference for cephalosporins and the mixed-mode of inhibition by captopril (Table 3 and Figure 5).

Conclusion

A metagenomic analysis has demonstrated that B3 MBLs are far more diverse and widespread than initially appreciated. In particular, uniquely among MBLs, members of the B3 subclass display four active site variations (11). The most common variant is characterized by the HHH/DHH motif for the Zn1/Zn2 metal ion binding sites. In the second most common variant, one histidine in the Zn1 site is replaced by a glutamine resulting in the QHH/DHH motif. Members of both variants were previously investigated and demonstrated that the single active site variation was found not to result in significant catalytic changes (46). In the rarest and also most divergent variant three of the six active site ligands are replaced, including one in the Zn1 site (a histidine by an arginine) and, uniquely, two in the Zn2 site (two histidines by a glutamine and a lysine) resulting in the HRH/DQK motif. In this variant the catalytic and metal binding properties are significantly impacted when compared to other B3 MBLs (11). Importantly, it could be shown that the variations in the Zn2 site, especially the replacement of a histidine to a lysine in position 263, were pivotal to these observed changes.

A phylogenetic analysis has also revealed the presence of a fourth active site variant among the B3 MBLs (11). Slightly less abundant than the B3-Q subclass, in this variant His116 in the Zn1 site is replaced by a glutamate resulting in the EHH/DHH motif. In agreement with the reported observation that variations in the Zn2 site exert a more profound effect on the properties of B3 MBLs than variations in the Zn1 site (11), the catalytic properties of SIE-1, the first example of a B3-E variant, are comparable to those of canonical B3 MBLs. However, the enzyme is as at least as efficient in hydrolyzing cephalosporin antibiotics as penicillin derivatives, which is rarely observed among the B3 MBLs (Table 3).

The high-resolution crystal structure of SIE-1 illustrates the conserved geometry characteristic for MBLs (Figures 2 and 5), but also confirms the terminal, monodentate coordination of Glu116 (Figures 2 - 4), the first example of an MBL with a glutamate ligand. The crystal structure also demonstrates that the N-terminal end is in the vicinity of the active site, thus forming a substrate binding pocket that is deeper than in many other MBLs. This conformation may contribute to the preference of SIE-1 for cephalosporins but may also be the reason why the inhibitor

captopril can adopt a competitive and uncompetitive binding mode compared to the usual purely competitive mode observed in MBLs (18, 60, 65).

In summary, here we describe the characterization of the first representative of an MBL from the B3-E subclass. Although this enzyme largely behaves like canonical and well-characterized MBLs, its rare preference for cephalosporins and occurrence in an opportunistic pathogen may be cause for some concern in the health sector. It is thus important to include SIE-1 in future inhibitor design studies as its interaction(s) with such compounds may inform important fine-tuning strategies to widen the therapeutic scope of much needed anti-resistance drugs. For example, the presence of an active site-bound glycerol can serve as the basis for the structure-based design of suitable inhibitor candidates.

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Table 1. Active site variations within the B3 MBL subclass. Only the ligands for the metal ions in the Zn1 and Zn2 sites are shown. Sequence positions are shown in subscript and variations from the canonical motif are highlighted in bold.

Variant	Zn1 site	Zn2 site
Canonical	$H_{116}H_{118}H_{196}$	$D_{120}H_{121}H_{263}$
B3-**Q**	$\mathbf{Q}_{116}H_{118}H_{196}$	$D_{120}H_{121}H_{263}$
B3-**E**	$\mathbf{E}_{116}H_{118}H_{196}$	$D_{120}H_{121}H_{263}$
B3-**RQK**	$H_{116}\mathbf{R}_{118}H_{196}$	$D_{120}\mathbf{Q}_{121}\mathbf{K}_{263}$

Table 2. Crystallographic refinement parameters for SIE-1. Values in brackets indicate the highest resolution shell.

Data collection parameters	
Resolution Range (Å)	48.19 - 1.68 (1.71 - 1.68)
Observations (I>σ(I))	420140 (19664)
Unique reflections (I>σ(I))	31438 (1534)
Completeness (%)	99.9 (97.2)
Mean <I/σ(I)>	20.1 (2.8)
[b]R_{merge}	0.069 (0.669)
[c]$R_{p.i.m.}$	0.020 (0.190)
Multiplicity	13.4 (12.8)
Space group	$P\,2_1\,2_1\,2$
Unit cell lengths (Å)	a=62.639 b=75.421 c=56.675
Unit cell angles (°)	α= β= γ= 90
Refinement statistics	
R_{work}	0.1414 (0.1612)
R_{free}	0.1697 (0.2082)
rmsd bond lengths (Å)	0.02
rmsd bond angles (°)	1.55
[d]Clash score	6.4
Ramachandran plot statistics	
Favored regions	96.97
Outlier regions	0
Rotamer outliners	0
PDB code	7LUU

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Table 3. Kinetic parameters for SIE-1 and representatives B3 MBLs containing the canonical active site motif (L1 and AIM-1), or the B3-Q (GOB-1) and B3-RQK (CSR-1) variants for the hydrolysis of representative penicillin (penicillin G, ampicillin), cephalosporin (cephalothin, cefuroxime) and carbapenem (meropenem, imipenem) substrates. Units for k_{cat} and K_M values and k_{cat}/K_M ratio are s^{-1}, μM and s^{-1}/mM, respectively. Also included are corresponding data for the B1 MBL NDM-1 for comparison.

Substrate	SIE-1			L1[a-c]			AIM-1[d,e]			GOB-1[f]			CSR-1[g]			NDM-1[h,i]		
	k_{cat}	K_M	k_{cat}/K_M	k_{cat}	K_M	k_{cat}/K_M	k_{cat}	K_M	k_{cat}/K_M	k_{cat}	K_M	k_{cat}/K_M	k_{cat}	K_M	k_{cat}/K_M	k_{cat}	K_M	k_{cat}/K_M
Penicillin G	360 ± 20	450 ± 50	790	410	75[a]	5470	778	31	25000	630	190	3400	17	250	70	11	16[g]	680
Ampicillin	100 ± 20	1900 ± 600	50	580	300[a]	1930	594	41	14000	ND	ND	ND	11	178	60	15	22[g]	660
Carbenicillin	99 ± 6	250 ± 50	400	ND	ND	ND	ND	ND	ND	ND	ND	ND	1	210	5	108	285[h]	380
Meropenem	92 ± 6	100 ± 20	910	77	13[c]	5920	1000	163	6100	170	22	8000	6	310	20	12	49[g]	250
Imipenem	27 ± 3	250 ± 60	110	384	48[b]	8000	1700	97	18000	85	13	6500	8	276	30	20	94[g]	210
Biapenem	1 ± 0.2	150 ± 50	7	64	75[a]	850	235	290	850	ND	ND	ND	9	298	30	30	120[h]	250
Cephalothin	16 ± 1	13 ± 1	1260	65	43[a]	1510	529	38	14000	32	7.9	4000	ND	ND	ND	4	10[g]	400
Cefuroxime	25 ± 2	25 ± 5	990	53	130[a]	410	292	29	10000	ND	ND	ND	1.0	110	10	5	8[g]	610
Nitrocefin	29 ± 3	32 ± 5	900	31	12	2600	240	96	2500	14	16	870	2	74	30	10	15[h]	670

[a-c]Data for L1 were reported previously by Crowder *et al.*, Simm *et al.* and Spencer *et al.* (47, 48, 66); [d]Data for AIM-1 were reported previously by Yong *et al.,* and Selleck *et al.* (43, 67); [f]Data for GOB-1 were reported previously by Horsfall *et al.* (13); [g]Data for CSR-1 were reported previously by Pedroso *et al.* (11); [h]Data for NDM-1 were reported previously by Yong *et al.,* and [i]Marcoccia *et al.* (68, 69). ND not determined. Data were measured in triplicate and variation is reported as standard errors.

Figure 1. Inhibitory effect of captopril on the catalytic rate of SIE-1. Catalytic rates were measured using the substrate meropenem. The data were fit using the mixed model inhibition equation (35).

Figure 2. The overall and active site structure of SIE-1. (A) Overall ribbon structure of SIE-1 with α-helices shown in red, loops in orange and β-sheets in green. Zn^{2+} ions are shown in grey, and residues discussed in the text are shown with carbon atoms in green, nitrogen atoms in blue, and oxygen atoms in red. (B) Active site structure of SIE-1 showing Zn^{2+} ions in grey, water molecules (Wat1, Wat2) in red, and metal-ligand interactions in yellow. The bound glycerol is shown with carbon atoms in cyan and oxygen atoms in red. The $2F_o$-F_c electron density for glycerol is shown as blue chicken wire and is contoured at 1.7 σ.

Figure 3. The structure of the canonical B3 MBL SMB-1 with hydrolyzed imipenem bound to the active site. Zn^{2+} ions are shown in grey, water ions in red. Residues are shown with carbon atoms in green, oxygen atoms in red, and nitrogen atoms in blue. The bound imipenem is shown with carbon atoms in cyan, oxygen atoms in red, nitrogen atoms in blue and sulfur atoms in yellow. Metal-ligand interactions are shown as yellow dotted lines.

Figure 4. Active site structures of SIE-1 and a canonical B3 MBL showing interactions between bound molecules and secondary sphere residues. (A) Active site of SIE-1 showing interactions between the bound glycerol, Zn^{2+} ions and secondary sphere residues involved in substrate binding. Note that Asn223 is fitted as two alternative conformations (69% and 31% occupancy). (B) Active site of SMB-1 showing interactions between the bound hydrolyzed imipenem, Zn^{2+} ions and secondary sphere residues. Note Ser221 is fitted as two alternative conformations (56). In both structures, Zn^{2+} ions are shown in grey, water molecules in red, nitrogen atoms in blue, oxygen atoms in red, and sulfur atoms in yellow. Residues are shown with carbon atoms in green while the bound glycerol and imipenem are shown with carbon atoms in cyan. Intermolecular interactions are shown as yellow dotted lines.

739 Figure 5. Comparison of the structures of (A) SIE-1, (B) Rm3 (PDB 5IQK), (C) L1
740 (PDB 1SML) and (D) AIM-1 (PDB 4AWY) showing the relative lengths of the
741 structurally resolved N-terminal ends (61, 63, 64). Zn^{2+} ions are shown in grey, N-
742 terminal tails are displayed in blue. Trp41 is shown in all structures as blue liquorish
743 sticks (61, 63, 64).
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