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β-lactamases inactivate the important β-lactam antibiotics by catalysing the hydrolysis of the β-lactam ring, thus. One class of these enzymes, the metallo-β-lactamases, bind two zinc ions at the active site and these play important roles in the catalytic mechanism.
A Variety of Roles for Versatile Zinc in Metallo-β-Lactamases

A. I. Karsisiotis, C. F. Damblon, G. C. K. Roberts

Metallo-β-lactamases are important as a major source of resistance of pathogenic bacteria to the widely used β-lactam antibiotics. They show considerable diversity in terms of sequence and are grouped into three subclasses, B1, B2 and B3, which share a common overall fold. In each case the active enzyme has binding sites for two zinc ions in close proximity, although the amino-acid residues which coordinate the metals vary from one subclass to another. In subclasses B1 and B3, there has been controversy about whether both zinc ions are required for activity, but the most recent evidence indicates that there is positive cooperativity in zinc binding and that the catalytically relevant species is the di-zinc enzyme. Subclass B2 enzymes, on the other hand, are active in the mono-zinc state and are inhibited by the binding of a second zinc ion. Evidence for the importance of the zinc ions in substrate binding has come from structures of product complexes which indicate that the β-lactam core binds to subclass B1 and B3 enzymes in a rather consistent fashion, interactions with the zinc ions being centrally important. The zinc ions play key roles in the catalytic mechanism, including facilitating nucleophilic attack on the amide carbonyl by the zinc-bound hydroxide ion, stabilising the anionic tetrahedral intermediate and coordinating the departing amine nitrogen.

The importance of metallo-β-lactamases

β-lactam antibiotics are an essential part of the arsenal of antibacterial chemotherapy and include structures of considerable diversity, including penicillins, cephalosporins, monobactams and carbapenems; examples are shown in Figure 1. β-lactams act as pseudosubstrates for DD-transpeptidases, also known as penicillin binding proteins (PBPs). The cleavage of the β-lactam bond leads to acylation and inhibition of the transpeptidases, thus irreversibly inhibiting the formation of a crosslinked peptidoglycan wall, decreasing its mechanical strength and leading to lysis. Bacteria have evolved three strategies to escape the activity of β-lactam antibiotics: alteration of the target site (PBPs), reduction of drug permeation across the bacterial membrane and, most importantly, production of β-lactamase enzymes. These enzymes catalyze the hydrolysis of the four-membered heterocyclic ring with cleavage at the amide bond. In the sequence-based Ambler classification of β-lactamases, classes A, C and D are evolutionarily distinct but all function with an active site serine residue whose side chain is the key nucleophilic agent in the catalytic process. Class B represents the zinc-dependent metallo-β-lactamases (EC 3.5.2.6; MBLs).

The zinc-dependent MBLs have no structure or sequence similarity to the serine β-lactamases and have a broad substrate profile, acting on most of the β-lactam scaffolds illustrated in figure 1. Zinc-dependent β-lactamases were first discovered as early as 1966 by Sabath & Abraham in an innocuous strain of Bacillus cereus. More chromosomally encoded metallo-β-lactamases were identified in the 1980s, but the discovery of transferable MBLs in the 1990s, first identified in Pseudomonas aeruginosa and Bacteroides fragilis, alarmed the scientific and clinical communities, since for the first time, the carrier organisms were clinically relevant and the possibility of widespread horizontal dissemination of these enzymes was recognised. A large number of transferable MBLs have been identified, including the large IMP and VIM families and most importantly NDM-type enzymes, all of which have spread worldwide and which are of major clinical and public health concern.

In this article we summarise recent work on this interesting and important family of enzymes, with the emphasis on the binding of zinc and its role in substrate binding and in the catalytic mechanism. We have not been able to cover the extensive literature comprehensively; the reader is referred to recent reviews for additional information. The MBLED database lists >600 entries.

Sequence and overall structure of MBLs; the three subclasses

MBLs are between 240-310 amino-acids in length, of which 17-30 amino-acids are signal peptides which are removed from the mature proteins. In order to facilitate the analysis of the available structures and the catalytic mechanisms a standard numbering scheme for class B β-lactamases, the BBL numbering, is widely used.
Figure 1: Scaffolds and representative examples of different types of β-lactam compounds. The β-lactam scaffold is highlighted in red and the numbering scheme has been included for Penicillins, Cephalosporins and Carbapenems.

Table 1
Structural and sequence diversity of the three subclasses of MBL.

<table>
<thead>
<tr>
<th></th>
<th>Subclass B1</th>
<th>Subclass B2</th>
<th>Subclass B3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structures</td>
<td>BcII, CerA, IMP-1, BlaB, IND-7, VIM-2, VIM-4, VIM-7, VIM-31, SPM-1, GIM-1, NDM-1</td>
<td>CphA, SFH-1</td>
<td>L1, FEZ-1, BJP-1, SMB-1, AIM-1</td>
</tr>
<tr>
<td>Metal Coordination</td>
<td>H116, H118, H196 (Zn1 site) D120, C221, H263 (Zn2 site) or H116, H118, H196 (Zn1 site, monozinc)</td>
<td>D120, C221, H263 (Zn2 site, monozinc) N116, H118, H196 (Zn1 site, inhibitory)</td>
<td>H116, H118, H196 (Zn1 site) D120, H121, H263 (Zn2 site) or Q116, H118, H196 (Zn1 site) D120, H121, H263 (Zn2 site)</td>
</tr>
<tr>
<td>Sequence Identity (within subclass)</td>
<td>20-99%</td>
<td>55-96%</td>
<td>21-94%</td>
</tr>
<tr>
<td>Sequence Identity (between subclasses)</td>
<td>14-24% with B2 2-15% with B3</td>
<td>14-24% with B1 2-16% with B3</td>
<td>2-15% with B1 2-16% with B2</td>
</tr>
</tbody>
</table>
MBL sequences and >150 crystal structures (including ligand complexes and mutants). MBLs are grouped into three subclasses, B1, B2 and B3 (Table 1) according to sequence similarities and zinc coordination, although sequence similarities are not large. Sequence identity between subclass B1 and B2 enzymes ranges from 14 to 24%, while sequence identity between subclass B3 and (B1+B2) enzymes ranges from 2% to 14%. The key sequence markers are the metal coordination ligands, which are conserved in a consensus motif of HXHXD(X)_iH(X)_jC(X)_kH (where i=55-74, j=18-24 and k=37-41). Phylogenetic analysis suggests that subclasses B1 and B2 are evolutionarily related and share significant sequence and structural similarities, but subclass B3, while sharing structural similarities with B1 and B2, is evolutionarily much more distant. It has been proposed that a revised classification scheme would be more correct, with only two subclasses, (B1+B2) and B3, but there are clear mechanistic and specificity differences between enzymes of subclasses B1 and B2 (see below).

The identification of the three subclasses is greatly strengthened by structural data. All MBLs for which a three-dimensional structure has been determined (listed in the MBLED database) share a common four-layered αβ/βα structure, with a central β-sheet sandwich flanked on either side by α-helices, the so called MBL-fold (Figure 2). This was first seen in MBLs, but has since been observed in a range of other proteins, with a range of different catalytic activities and metal specificities. Most of these fall into two groups: (i) hydrolases, including MBLs themselves, glyoxalase II, lactonases and nucleases (such as RNase Z), involved in tRNA maturation, and Artemis, involved in V(D)J recombination), which generally have di-zinc or di-manganese metal centres, and (ii) oxidoreductases, with di-iron metal centres, such as rubredoxin:oxygen oxidoreductase, nitric oxide reductase and a β-hydroxylase involved in antibiotic biosynthesis. The ability of this structural scaffold to serve multiple catalytic functions, at least within one of these groups, has been directly shown by the demonstration that extensive protein engineering of human glyoxalase II could produce a functional MBL.

![Figure 2: Representative MBL Structures. Subclass B1 (BclI, IMP-1, VIM-2, NDM-1 and SPM-1), subclass B2 (CphA and SFH-1) and subclass B3 (L1, AIM-1 and SMB-1). PDB identifiers are given for each structure.](image)
Structural features and substrate specificity

The prototypical subclass B1 MBL, BcII (Figures 2 & 3) is comprised of 227 residues in the mature form, in the αβ/βα MBL fold, a sandwich of seven (β1β2β3β4β5 antiparallel, β5β6β7 parallel) and five (β8β9β10β11 antiparallel, β11β12 parallel) β-sheets. The α-helices are surface exposed, two flanking each of the two domains (N-terminal residues 1-120 and C-terminal residues 129-227), with a fifth from the N-terminal domain (α3, 109-117) located on the external face in between the two domains. The active site, with the two zinc ions, is at one edge of the β-sheet sandwich, in a relatively deep cavity formed by two long flanking loops (residues 60–66 and 223–241; residue numbers are those in the standard BBL system\(^7\)). B1 MBLs have broad substrate specificity, hydrolysing penicillins, cephalosporins and carbapenems. There are, however, exceptions: they do not act on monobactams, and BcII does not hydrolyse talampicillin, which has an additional substituent on C7, or temocillin, in which the C3 carboxylate is replaced by a bulky substituent\(^\text{20}\) (see Figure 1), while VIM7 has decreased activity relative to VIM-2 towards cephalosporins\(^\text{21}\), probably attributable to substitutions in the mobile loop composed of residues 60-66 (see below).

The shape of the binding site differs between enzymes of the three subclasses and to a lesser extent between enzymes of the same subclass. This is illustrated in Figure 3 by cones having their tips on the zinc ions and dimensions defined by the van der Waals surface. The structurally most divergent of the subclass B1 enzymes whose structure is known is SPM-1\(^\text{22}\) (Figure 2). This enzyme lacks the mobile loop (residues 60-66) implicated in substrate binding (see above) and a central insertion of 24 amino-acids is accommodated in an extended helical region between the two domains, although deleting this had only relatively modest effects upon hydrolysis of a range of β-lactams\(^\text{22}\).

Figure 3: Active site cavities of typical subclass B1 (BcII, 1BVT), B2 (CphA, 1X8G) and B3 (FEZ-1, 1KO7) enzymes, together with an indication of the typical substrate specificities of enzymes of each subclass (see, e.g.\(^\text{2a, 20, 23}\)). The imaginary cones which can be formed in the opening formed from the zinc ion to the protein surface, are depicted. Figure based on one from\(^\text{24}\).
Enzymes of the B2 subclass (Figure 2) are strict carbapenemases; as described below, they are active with only a single zinc ion. The overall fold is the same characteristic MBL fold, but the active site groove is deeper and narrower, perhaps contributing to the narrow substrate profile of these enzymes. The α3 helix (R140-L161) is elongated relative to the corresponding helix in B1 enzymes, and has an unusual kink (around W150) enabling it to follow the curvature of the enzyme’s surface in the immediate vicinity of the active-site groove and provide a surface which contributes to substrate binding. On the other hand, subclass B2 enzymes lack the mobile loop (residues 60-66) observed in the structures of subclass B1 enzymes, which contributes to substrate binding. From an evolutionary perspective, it is interesting that the MBL SPM-1 also lacks the mobile loop and has an elongated α3 helix, although in terms of sequence and substrate specificity it clearly belongs to subclass B1.

In subclass B3 MBLs (Figure 2), which include the only known tetrameric MBL, L1, the differences from subclass B1 enzymes include an elongated C-terminal helix and longer α3-β7 and β12-α5 loops. By contrast, the equivalent of the β3-β4 (60-66) loop in subclass B1 enzymes is a significantly shorter (by 7-8 residues) β2-β3 loop and supporting β-sheets. Another distinct characteristic is the presence of an intramolecular disulphide bridge, which constrains the elongated β12-α5 loop and stabilises the elongated C-terminal helix. The tetramerization of L1 is attributed to hydrophobic interactions involving the sidechain of the non-conserved M175 in a hydrophobic pocket comprising L154, P198, and Y236; mutation of M175 to aspartate led to the formation of a monomeric enzyme with significantly decreased catalytic activity.

There are several structural differences between enzymes of subclass B3. For example SMB-1 has a shortened N-terminus, while BJP-1 has an N-terminal α-helix, residues of which (e.g. F31) restrict the active site cavity resulting in low affinity for β-lactams. SMB-1 and AIM-1 have a non-conserved residue (Q157) which contributes to β-lactam binding. The conformations of two active site loops, residues 156-162 and 223-230, are also variable; in SMB-1 and AIM-1, these loops are spatially closer together than other B3 enzymes.

Metal coordination

MBLs require zinc as a metal cofactor and have two zinc binding sites in close proximity to one another; these are often referred to as Zn1 or the 3H site and Zn2 or the DCH site, although there are variations in the coordinating residues, particularly between the different subclasses (Table 1). In a number of cases crystal structures have been obtained with a single zinc ion bound (particularly when crystallised at low pH), but all the evidence indicates that all MBLs can bind two zinc ions under the right conditions. Representative coordination structures are shown in Figure 4. There has been controversy about the number of zinc ions required for catalysis and the mechanism of metal binding; this is discussed below in the context of the individual subclasses.

MBLs function in vivo only with zinc, but in vitro significant activity is seen with other metal ions, including Cd(II), Co(II), Ni(II), Cu(II), and Mn(II) (e.g. 29). In two cases, the cadmium-substituted B. fragilis enzyme and the cobalt-substituted B. cereus enzyme, both members of the B1 subclass, the metal substitution has very little effect on the coordination geometry. The use of metal substitution has proved a valuable tool in the study of MBLs, notably by allowing the use of spectroscopic probes – including electronic, EPR, NMR and perturbed angular correlation (PAC) spectroscopy (e.g. 29c, 32) – which are not available for the native zinc enzyme. Examples will be referred to briefly in the discussions below.

Subclass B1.

In the MBLs of subclass B1 the coordination ligands of the two zinc sites are highly conserved. The first site (Zn1) is composed of H116, H118 and H196 (BBL numbering) in a tetrahedral coordination with the fourth ligand being a water or hydroxide bridging to the Zn2. The second site (Zn2) is composed of an aspartate (D120), a cysteine (C221) and a histidine (H263) – an unusual combination of ligands for zinc in a protein – in a distorted
trigonal bipyramidal coordination with an apical water molecule (or sometimes a carbonate ion) and the bridging water/hydroxide as fourth and fifth ligands (Figure 4, top right). The presence of a cysteine ligand for Zn2 appears to be characteristic of MBLs; in other members of the MBL-fold superfamily, the homologous residue is an aspartate\(^2\). Interestingly, in BcII and IMP-1 the C221D mutants are fully active, but only in the presence of excess zinc, indicating weaker metal binding\(^3\).

In two structures, of IND-7 and VIM-2, the arrangement of water molecules in the active site leads to differences in zinc coordination: an additional water weakly bound to Zn1 leads to a pentacoordinate trigonal bipyramidal geometry, while the apical water molecule is missing from Zn2, leading to a tetracoordinate tetrahedral geometry\(^5\), clearly illustrating the flexibility of zinc coordination in MBLs. In crystal structures having only a single zinc ion bound, this is located in the Zn1 site, coordinated by three histidines and a water molecule (Figure 4, top left). The relative macroscopic dissociation constants for zinc binding to the two sites vary considerably between different MBLs and under different solution conditions.

It is notable that the zinc-zinc distance is quite variable, ranging from 3.5 Å to 4.6 Å even when comparing different structures of the same enzyme. Comparison of structures obtained at different pH values, supported by molecular dynamics simulations, suggests that this variability reflects the difference between a bridging water (\(r_{\text{Zn-Zn}} \sim 4.5\) Å) and a bridging hydroxide ion (\(r_{\text{Zn-Zn}} \sim 3.5\) Å)\(^6\). Three crystal structures of enzymes of the B1 subclass having a single zinc ion bound have been reported\(^2, 7\), and there has been considerable controversy about the catalytic activity of the mono-zinc enzymes. It is clear that when the enzymes are assayed in the presence of a stoichiometric quantity of 1 mole zinc per mole of enzyme, substantial catalytic activity is indeed observed; addition of excess zinc leads to a significant, though not dramatic, increase in activity. This led to the proposal that only a

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**Figure 4:** Representative zinc coordination examples. Di-zinc BcII (1BVT), mono-zinc BcII (1BMG), mono-zinc CphA (1X8G) and di-zinc L1 (1SML). BBL numbering is used for all examples.
single zinc ion is required for activity, and mechanisms which depend on only one zinc ion have been formulated (see below).

However, there is now good evidence in both the *B. cereus* and *B. fragilis* enzymes for substantial positive cooperativity in zinc binding. In the case of the *B. fragilis* enzyme, which binds both zinc ions tightly, Fast et al. prepared an enzyme containing one molar equivalent of zinc and found that the Km for nitrocefin was virtually unchanged, while kcat was decreased by a factor of two. Studies of the pre-steady-state kinetics of nitrocefin hydrolysis, during which an anionic intermediate is observed (see below) showed that the experimental data for the one-zinc preparation could be reproduced by the same kinetic mechanism and rate constants determined for the native two-zinc enzyme, but with the enzyme concentration reduced by 50%. This is clearly consistent with the idea that the one-zinc preparation is in fact a mixture of di-zinc and apo-enzyme, and hence with the existence of positive cooperativity in zinc binding. In the case of the BcII enzyme (from *B. cereus* 569/H/9) direct physical evidence for this cooperativity has been obtained. ESI mass spectrometry of the enzyme during titration of the apo-enzyme with zinc showed that >10% di-zinc enzyme was present at Zn:enzyme < 0.5, and the maximum level of the mono-zinc species was ≤20%, providing direct evidence for positive cooperativity. ^38b, 39^ 1H–15N NMR spectroscopy experiments allow observation of the enzyme at the level of individual backbone NH groups and histidine imidazole NH and N(C)H groups – including the zinc-coordinating histidines. Upon zinc titration, signals characteristic of the di-zinc enzyme were readily observed from the beginning of zinc addition. At molar ratios Zn:enzyme in the range 0.2-1.8, resonances from both the apo- and the di-zinc enzymes were present but no other species could be detected, the detection limit in the NMR experiments being ~10% ^38b^.

Measurements of catalytic activity during the zinc titration showed a linear increase from zero to full activity at Zn:enzyme = 2; taken together with the MS and NMR experiments this strongly suggests that zinc binding shows positive cooperativity and that the di-zinc species is the only relevant one for catalysis. ^38b^.

This is also consistent with the kinetic and calorimetric results of Badarau and Page. ^40^ It is interesting to note that titration of the apo-enzyme with cadmium rather than zinc gives quite different results. In both the MS and NMR experiments the mono-cadmium species is readily observed; indeed in the MS experiments this is the only species observed at Zn:enzyme = 1 ^38b^. This is an important control for the observations with zinc, but also illustrates the fact that different metal ions binding to the same sites in an apparently unchanged protein structure can behave quite differently. Indeed the combination of NMR & PAC spectroscopy and measurements of macroscopic dissociation constants was used to suggest that cadmium shows negative cooperativity in binding to BcII. ^32b^ Similarly, detailed spectroscopic studies of the BcII enzyme provide clear evidence for the formation of a mono-cobalt species which appears to be catalytically active. ^41^.

**Subclass B3**

The crystal structures of MBLs of the B3 subclass ^26^ show them to be di-zinc enzymes (the L1 enzyme is, uniquely, a tetramer, but its zinc coordination (Figure 4, bottom right) is essentially identical to that seen in FEZ-1 ^26b^). In the Zn1 (3H) site the metal ion is coordinated by the same conserved residues (H116, H118 and H196, BBL numbering) as subclass B1 enzymes, and by a bridging water (or hydroxide ion). By contrast, while the distorted trigonal bipyramid geometry of the Zn2 site is retained, the ligands comprise aspartate (D120) and histidine (H263), equivalent to those seen in subclass B1 enzymes, together with a second, non-conserved, histidine residue (H121), the bridging water molecule and a second, apical, water. The serine (S221) which replaces the cysteine ligand in B1 enzymes is not involved in metal binding. The aspartate and the second water are apical ligands, as in B1 enzymes; however in the B3 enzymes the positions of the three planar ligands, the two histidines and the bridging water, have moved such that the entire plane has undergone a rotation of approximately 80° about the bond between the bridging water and Zn2. Interestingly, proteins other than MBLs which share the MBL fold
share B3-like metal coordination topology, but with an additional aspartate residue which bridges the two metal ions (see, e.g., \(2^\text{a}\)).

An exception among the subclass B3 enzymes is provided by the family of GOB enzymes from the clinically important organism Elizabethkingia meningoseptica; in these enzymes the coordinating residue His116 in the Zn1 site is replaced by a glutamine. In the case of the GOB-18 enzyme, evidence has been presented for an active mono-zinc enzyme, in which the metal is bound in the Zn2 site\(^\text{42}\); studies of the binding of other metals provide good evidence against a di-metal enzyme\(^\text{42-43}\), but in the case of zinc this possibility cannot yet be definitively excluded. Indeed, studies of the GOB-1 enzyme (which differs from GOB-18 by only three amino-acid substitutions, far from the active site) showed that the enzyme as isolated contained two bound zinc ions\(^\text{44}\). As yet, no structure is available for an MBL of this family and this discrepancy remains to be resolved.

### Subclass B2

Enzymes of this subclass are functionally distinct from those of subclasses B1 and B3 in two ways: (i) they are strict carbapenemases and show very poor activity against penicillins and cephalosporins; (ii) while the activity of subclass B1 and B3 enzymes increases when zinc is added beyond a molar ratio of 1:1 (see above), enzymes of subclass B2 are inhibited in a non-competitive manner by the binding of a second zinc ion\(^\text{45}\). Crystal structures of the Sfh-1 enzyme from Serratia fonticola\(^\text{25a}\) and of the CphA enzyme from Aeromonas hydrophila\(^\text{25b}\) both show a single zinc ion bound in the Zn2 (DCH) site (Figure 4, bottom left), with the erstwhile Zn1 site occupied by water molecules. This is consistent with EXAFS (extended X-ray absorption fine structure) studies and with the observation of sulphur-to-metal charge transfer bands in the electronic spectra of the Cu(II) and Co(II) substituted CphA enzyme\(^\text{46}\) but is in contrast to the structures of mono-zinc B1 enzymes, where the single zinc ion is bound in the 3H site. The structure of the di-zinc CphA enzyme\(^\text{47}\) shows that the second zinc ion is bound in a modified Zn1 site; His118 and His196 are conserved, but the third ligand His116 is not conserved and is replaced by an asparagine residue, which is not involved in metal binding. It has been proposed\(^\text{47}\) that the binding of the second zinc ion inhibits B2 enzymes by immobilising His118 and His196 and preventing them from playing their roles in the postulated catalytic mechanism\(^\text{25}\) (see below) and perhaps also by inducing a small movement of the Gly-232-Asn233 loop, hindering substrate access to the active site.

### Substrate binding – role of metals and of active site residues

#### Substrate specificity

The MBLs belonging to subclasses B1 and B3 are broad spectrum enzymes and hydrolyze most \(\beta\)-lactam antibiotics including carbapenems (Figure 3; Bebrone\(^\text{2a}\) and references therein). Subclass B1 enzymes act efficiently on penicillins, cephalosporins and carbapenems, while subclass B3 enzymes preferentially hydrolyse penicillins and cephalosporins but are not as efficient on carbapenems. Modification of the carboxylate group at the C3 position in penicillins or C4 position in cephalosporins reduces the susceptibility to hydrolysis. The subclass B2 enzymes are strict carbapenemases and show a very weak activity, if any, towards penicillins and cephalosporins. Interestingly, a mutant of CphA, N116H-N220G, in which the asparagine in the Zn1 site and the asparagine immediately preceding the cysteine in the Zn2 site were replaced by the equivalent residues in B1 enzymes, showed a considerably broadened activity spectrum being able to hydrolyze penicillins and cephalosporins efficiently in addition to carbapenems (although with a reduced efficiency towards the latter)\(^\text{48}\). Only the monobactams are resistant to hydrolysis by MBLs, and bind only weakly and non-productively\(^\text{49}\). In addition, MBLs are not inhibited by classical inhibitors of serine \(\beta\)-lactamases such as clavulanic acid, sulbactam and tazobactam; these compounds in fact behave as poor substrates.

The importance of the zinc ions for substrate binding is highlighted by the fact that the BeII apo-enzyme cannot bind substrate, but the ability to do so is restored upon reconstitution of the di-zinc enzyme\(^\text{50}\).
Inhibitor binding
A good deal of structural information on inhibitor binding is available from crystallography and NMR spectroscopy (see for example, thiol52, carboxylate53, or tetrazole54 groups, and thiol-containing compounds show activity as broad-spectrum MBL inhibitors52, 55. However, the chemical structures of most of these inhibitors bear little resemblance to those of MBL substrates, and therefore the three-dimensional structures of their complexes provide little useful information on the mode of substrate binding.

One possible exception is the role of the two loops on either side of the active site. In particular, the ‘mobile loop’ or ‘flap’ (residues 60-66; BBL numbering), which is conserved in B1 MBLs, plays a role not only in inhibitor binding but also in catalytic activity56. With the exception of a glycine residue at position 63, none of the residues in this loop are fully conserved, but detailed mutagenesis studies56 demonstrated its importance for catalytic activity. The flexibility of this loop in the free enzyme has been shown by crystallography (e.g. 57) by NMR spectroscopy51, 58 and by computational methods59, although solution structural studies indicate that it does have a reasonably well-defined structure in the absence of substrate or inhibitor51. On inhibitor binding this loop moves towards the bound inhibitor, apparently by a hinge-bending kind of motion51. Very recently it has been shown that the 19F chemical shifts of a fluorinated label attached to a residue in this flexible loop are sensitive to the different modes of binding of different inhibitors. Studies of inhibitor binding (see and references therein), the effects of mutagenesis on the hydrolysis of different substrates56 and computational studies56b show that this loop is important for the formation of a hydrophobic pocket which is important for inhibitor and substrate binding.

Product binding
No direct structural information on substrate binding is available, since sufficiently long-lived enzyme–substrate complexes have not been identified, although a number of studies of docking substrates into MBL structures have been reported (e.g., 21, 26a, d, 57b, 61). In addition, importantly, a number of structures of product complexes of MBLs have been described. Of course the key atoms around the bond which is cleaved in the reaction will not be in the same place in these complexes, but they do allow one to identify a number of key interactions.

Subclasses B1 & B3. Valuable information on product (and hence substrate) binding to B1 MBLs was obtained from two studies on the NDM-1 enzyme62. Complexes with the hydrolysis products of four penams (benzyl penicillin, ampicillin, oxacillin and methicillin) show that the β-lactam core is bound in essentially identical way in all cases, with the R1 aromatic substituents showing significantly more variability in binding, and also a higher B-factor. All these complexes retain the bridging water/hydroxide in between the two zinc ions. A number of key interactions with groups on the product are conserved in all these structures (Figure 5): The C3 carboxylate substituent on the β-lactam ring is coordinated to Zn2 and forms an ion-pair with the conserved K224. This substituent is well-established as an important binding determinant. However, it is notable that, notwithstanding the observation of direct coordination in all complexes of products with B1 and B3 enzymes which have been studied, several QM/MM simulations of substrate binding have suggested that the interaction of this carboxylate with Zn2 is an indirect one, mediated by a water molecule63 though others support a direct interaction64. Monobactams such as aztreonam, which are resistant to the action of MBLs, lack the fused ring of the β-lactam core and hence lack this carboxylate group. Instead, they have a bulky sulfonate group attached to N4; this is displaced effectively one carbon unit from the carboxylate, and modelling indicates that it is too far away for direct coordination to Zn2 and has overall weakened electrostatic interactions with the enzyme active site62a. Zn2 is also coordinated by the N4 nitrogen of the hydrolysed β-lactam and by the carboxylate of D120. One oxygen of the C7 carboxylate formed by hydrolysis of the β-lactam appears...
to coordinate Zn1 in all cases (distance 2.4-2.5 Å), but the interaction of the other oxygen with the conserved N233 (BBL numbering) is more variable between the different complexes.

**Figure 5**: Enzyme – product complexes. A) NDM-1–hydrolyzed Ampicillin, B) NDM-1–hydrolyzed Benzyl Penicillin, C) NDM-1–hydrolyzed Meropenem, and D) L1–Moxalactam rearranged product. Protein coloured by atom type with carbon atoms in grey. Product coloured by atom type with carbon atoms in green. Interactions are depicted as follows: zinc coordination in black, product-zinc binding in red, hydrogen bonds in magenta and salt bridges in green. The figure was based on PDB files: (A) 3Q6X, (B) 4EYF, (C) 4EYL and (D) 2AIO

The hydrolyzed carbapenem substrate meropenem shows an interesting difference in its interaction with NDM-1 \(^{62a}\) (Figure 5). The interactions of Zn2 with the C3 carboxylate and with N4 are retained, but an oxygen of the C7 carboxylate directly coordinates both Zn1 and Zn2, acting as a bridging ligand, resulting in tetrahedral coordination of Zn1 and hexacoordination of Zn2 in the complex. In contrast to these strong interactions of the β-lactam core, the R2 group of meropenem (pyrrolidine N,N-dimethylcarboxamide) does not exhibit any strong interactions with NDM-1.

The structure of the complex of the rearranged hydrolysis product of the poor substrate moxalactam with the subclass B3 enzyme L1 \(^{65}\) shows a generally similar picture (Figure 5). Zn2 binds the C4 carboxylate and N5 of the oxacephem ring; the carboxylate displaces the ‘apical’ water from Zn2, and also interacts with S221 and S223, which are the functional equivalent of K224 in B1 enzymes. Zn1 is coordinated by an oxygen of the newly formed C8 carboxylate, changing from tetracoordinated to pentacoordinated.

All these structural studies of product binding are consistent with the importance of the loops on either side of the active site in substrate binding (as discussed above), notably in forming a hydrophobic pocket for the R1 substituent. It is very likely that the flexibility of these loops\(^{51,58}\) contributes to the ability of
Figure 6: CphA-Biapenem hydrolysis product. Structure of the CphA N220G mutant, complexed with hydrolyzed and rearranged Biapenem. Protein coloured by atom type with carbon atoms in grey. Product coloured by atom type with carbon atoms in green. Interactions are depicted as follows: Zinc coordination in black, product-zinc binding in red and hydrogen bonds in magenta. The backbone segment 232-233 of the superimposed wild type CphA structure is shown along with the sidechain of N233 in khaki. The figure was based on pdb files 1X8I and 1X8G.

MBLs to hydrolyse a structurally diverse range of substrates\textsuperscript{56, 59b, 66}.

**Subclass B2.** In view of the fact that the subclass B2 enzyme CphA is active only in the mono-zinc form, with the zinc ion bound in the Zn2 (DCH) site, one would expect substrate binding to be different from that seen in B1 and B3 enzymes. In fact, the crystal structure of the N220G mutant\textsuperscript{5} of CphA with the product of biapenem hydrolysis, where the initial hydrolysis product undergoes a substantial rearrangement to form a bicyclic structure, shows a considerable degree of similarity in the interactions of the zinc ion\textsuperscript{25b} (Figure 6). As seen in subclass B1 and enzymes, the zinc coordinates directly to the C3 carboxylate and to N4. This carboxylate also interacts with K224 and with the backbone NH of N233; there is a local change in backbone conformation involving N233-G232 to facilitate this latter interaction. In addition to the zinc ion, N4 interacts with a water molecule which in turn binds to H118 and D120; this water molecule has been implicated in the catalytic mechanism (see below). Finally, the second carboxylate group on the bicyclic structure interacts with two threonine residues.

**Catalytic mechanisms**

**Overview**

Zinc does of course have many advantages for involvement in enzyme catalysis which are manifest in MBLs, including its role as a Lewis acid, its flexible coordination geometry combined with strong binding to suitable sites – the four-coordinate 3H site and the five-coordinate DCH site – and fast ligand exchange – for example of the nucleophilic hydroxide. Even though zinc β-lactamases are much less studied than their serine counterparts, there is a consensus in terms of the general catalytic mechanism. The mechanism of MBLs has been extensively studied and well reviewed\textsuperscript{2b, 6a, 67}, and detailed studies of model reactions have been carried out\textsuperscript{68}. The likely roles of the zinc
ions are clear. The zinc acts as a Lewis acid by co-ordination to the amide carbonyl oxygen giving a more electron deficient carbonyl carbon which facilitates nucleophilic attack by the zinc-bound hydroxide and/or it stabilises the negative charge developed on the carbonyl oxygen of the tetrahedral intermediate anion. Coordination to the metal ion will lower the pKa of the co-ordinated water, increasing the concentration of the hydroxide species. However, the hydroxide ion formed by this ionisation, while it may become the dominant species, will be stabilised by simultaneous coordination to two zinc ions and hence will be more weakly nucleophilic\textsuperscript{68a, b, 69}. Finally, breakdown of the tetrahedral intermediate could be facilitated by direct co-ordination of the departing amine nitrogen to the metal ion or, alternatively, a metal-bound water could act as a general acid catalyst, protonating the amine nitrogen leaving group. A wide range of mechanistic studies have been carried out, including pH-dependence, isotope effects, the effects of mutagenesis (though due to the plasticity of the active site\textsuperscript{70} these are often difficult to interpret) and spectroscopic studies of metal-substituted enzymes, which demonstrated changes in metal coordination during catalysis\textsuperscript{71}. Nonetheless, distinguishing between the relative importance of these possible roles for zinc remains difficult.

**Two zinc mechanisms – subclasses B1 and B3**

Structural and mechanistic data on di-zinc B1 enzymes\textsuperscript{57, 61b, 72} have led to consistent mechanistic proposals, shown in Figure 7, and essentially identical mechanisms are likely to apply to subclass B3 enzymes\textsuperscript{6a}.

This mechanism involves the bridging hydroxide performing the nucleophilic attack on the \(\beta\)-lactam carbonyl carbon. It is not clear whether the carbonyl is polarised by an interaction with Zn1 to facilitate the attack by the hydroxide, as often implied, or whether the role of Zn1 is to form an ‘anion hole’ to stabilise the anionic tetrahedral intermediate. The majority of QM/MM calculations indicate that the interaction of Zn1 with the carbonyl oxygen takes place after the hydroxide attack, in the tetrahedral intermediate\textsuperscript{63a, b, 64b, c}, but in some calculations\textsuperscript{73} this interaction is present in the Michaelis complex. It should be noted that a substantially different mechanism has also been proposed\textsuperscript{36} in which the bridging water or hydroxide serves as a general base and the nucleophilic water originates from the bulk solvent. The oxyanion intermediate is further stabilized by interaction with the side chain of N233 (B1 enzymes) or Y228 (B3 enzymes), which may also polarise the carbonyl to promote the nucleophilic attack\textsuperscript{63a} (mutagenesis studies do not identify this residue as strictly essential in B1 or B3 enzymes\textsuperscript{74}).

![Figure 7](image)

**Figure 7**: Catalytic mechanism for di-zinc B1 MβLs (Based on Fink and Page\textsuperscript{67a}).

Zheng & Xu\textsuperscript{63b} suggest a concerted mechanism, with the nucleophilic attack by the bridging hydroxide, transfer of its proton to Asp120 and C-N bond cleavage occurring together. The role of D120 has been extensively discussed. It seems most probable that it has a structural role in positioning Zn2 and in orienting the bridging hydroxide; the results of mutagenesis experiments do not favour a role for this residue as a general base\textsuperscript{75} and the bridging hydroxide is already a good nucleophile\textsuperscript{64b}.
Substrate binding is facilitated by the interaction of the carboxylate with K224 and the binding of the large aromatic substituent with hydrophobic residues on the flap. Directed evolution experiments\textsuperscript{76} have shown that ‘second-shell’ residues can have significant effects on \( k_{\text{cat}} \), presumably by modulating these direct interactions.

Stopped-flow kinetic studies with the subclass B1 and B3 enzymes using the chromophoric \( \beta \)-lactam nitrocefin have provided evidence for the accumulation of an intermediate with a strong absorbance at 665\textnormal{nm}\textsuperscript{38a, 56, 77}. This was postulated to be due to the generation of a species in which the leaving nitrogen atom is not protonated on the cleavage of the amide bond and remains negatively charged, this anion being stabilized by extensive conjugation with the dinitrostyryl substituent in the C3 position and by interaction with Zn2 (Figure 8; note that an interaction between N4 and Zn2 has been commonly observed in structures of product complexes – see above). Further evidence for the formation of such an intermediate was obtained in studies of the hydrolysis of nitrocefin catalysed by a model zinc complex\textsuperscript{68c} and from computational work\textsuperscript{64b, 73}.

Breece et al.\textsuperscript{78} have used freeze-quench EXAFS to study the hydrolysis of nitrocefin by the L1 enzyme. The EXAFS of the species trapped after 10\textup{ms} reaction, a time corresponding to the maximum accumulation of the anionic intermediate, showed that the Zn-Zn distance had increased from 3.42\textnormal{Å} in the resting enzyme\textsuperscript{79} to 3.72\textnormal{Å}. Since Zn1 interacts with the C7 carbonyl and Zn2 with the C3/C4 carboxyl Breece et al.\textsuperscript{78} suggest that this

\textbf{Figure 8. Structure of the postulated intermediate in the MBL-catalysed hydrolysis of nitrocefin.}

motion, occurring on substrate binding or on movement of the bridging hydroxide, will exert strain on the C-N bond, helping to facilitate cleavage.

The accumulation of an anionic intermediate would imply that the protonation of this nitrogen leaving group, rather than the breaking of the C-N bond, is rate-limiting. However, this does not seem to be a general observation. Accumulation of the intermediate during nitrocefin hydrolysis is observed to varying extents for the CcrA, IMP-1 and L1 enzymes\textsuperscript{38a, 56, 77}, but not significantly for the BcII enzyme\textsuperscript{56}. In addition, nitrocefin is clearly an ‘abnormal’ \( \beta \)-lactam in which this anion would be unusually stable, and studies of other substrates in both the L1 and BcII enzymes have led to the conclusion that for the majority of \( \beta \)-lactams the breaking of the C-N bond is likely to be the rate-limiting step\textsuperscript{50, 80}.

The final step before product release is the protonation of the anionic intermediate. Two sources for this proton are possible: the apical water on Zn2 or a bulk water molecule stabilised and oriented by D120. In the former case, the implication would be that, in line with theoretical results (see above – Substrate Binding), in this intermediate Zn2 could not be coordinated directly by the C3 carboxylate. In all the structures of product complexes, the bridging water/hydroxide is present, even though this is consumed in the hydrolysis reaction and must be ‘replenished’. While it had been assumed that this occurred after product release, the presence of this water in the structures of product complexes suggests that replenishment may precede product dissociation\textsuperscript{62a}.

**One zinc mechanism – Subclass B1**

As discussed above crystal structures of enzymes of the B1 subclass with a single zinc ion bound have been reported\textsuperscript{22, 37}, and a mechanism for the catalytic activity of the mono-zinc enzymes has been formulated (Figure 9), having similarities to those of zinc peptidases such carboxypeptidase and thermolysin.

This proposed mechanism\textsuperscript{37a} involves deprotonation of a Zn-bound water molecule by D120, forming a hydroxide ion which performs the nucleophilic attack on the carbonyl carbon, the zinc ion stabilising the anionic tetrahedral intermediate, and the \( \beta \)-
lactam nitrogen receiving back the proton from D120. However, the pH-rate profile for the BcII-catalysed hydrolysis of benzylpenicillin and cephaloridine was taken to indicate that the zinc-bound water is fully ionized at neutral pH. It was suggested that nucleophilic attack by the zinc-bound hydroxide on the carbonyl is followed by a proton abstraction from the Asp120 to give a dianionic tetrahedral intermediate. In both cases, the zinc acts as a Lewis acid to stabilise this intermediate.

The physiological relevance of this mechanism for subclass B1 and B3 enzymes is unclear, in view of the evidence discussed above that for the majority of these enzymes the di-zinc enzyme is the dominant – and catalytically relevant – species due to cooperativity in zinc binding. The only known B1 and B3 MBLs which might function as mono-zinc enzymes are the GOB family and the SPM-1 enzyme; in both cases there remains uncertainty about the catalytically relevant species.

**One zinc mechanisms – subclass B2**

The first insights into the structural basis of the mechanism of subclass B2 enzymes, in which the single zinc ion is in the Zn2 (DCH) site were provided by the determination of the structure of the CphA enzyme in complex with hydrolysed and rearranged biapenem; a mechanism was proposed based on this structure and this has been further analysed by QM/MM simulations. In contrast to the mechanism proposed for mono-zinc B1 enzymes, where the zinc ion is in the Zn1 (3H) site, the nucleophilic attack is carried out by a water molecule which is not zinc-bound and

**Figure 9**: Proposed catalytic mechanism for monozinc B1 MβLs (Based on Fink and Page).

**Figure 10**: Catalytic mechanism for mono-zinc B2 MβLs (Based on 6a).
which is activated by D120 and/or H118; the structure of the Sfh-1 enzyme suggests that H118 rather than D120 is responsible for this activation, but computational studies implicate both residues. The zinc ion coordinates the β-lactam nitrogen and promotes the ring opening reaction by stabilizing the negative charge on nitrogen during the transition state; it is not involved in the interacting with the anionic transition state, which is stabilized by H196, D120 and H118. After a rotation about the C5-C6 bond, the newly formed carboxyl interacts with T119 and T157 and a water molecule protonates the lactam nitrogen, followed by release of the substrate. Computations suggest that the pathway leading to the rearranged product of biapenam hydrolysis is a kinetically unfavoured one, although the complex with this product is thermodynamically stable; it has been proposed that the rearrangement of the product takes place off the enzyme, followed by re-binding.

Two alternative mechanisms have been proposed which differ in detail from that shown in Figure 10. Simona et al. have considered as a starting point a Michaelis complex in which the C3 carboxylate is bound indirectly to the zinc ion through a water molecule; this water molecule is then positioned to donate a proton to the anionic N4 formed on cleavage of the C-N bond. They also propose that this water moves to replace the 'nucleophilic' water bound to H118 and D120, so that this mechanism is consistent with the observation of direct carboxylate-zinc coordination in the product complex. Ackerman & Gatti proposed that the nucleophile is not the water bound to H118 and D120 but rather a separate water molecule, activated by the H118/D120 water.

Conclusions
The dissemination of metallo-β-lactamases through transferable genetic elements makes these enzymes an important public health issue. In addition, in view of the diversity of functions supported by the MBL fold, they are of substantial evolutionary interest. While the overall fold is conserved in all MBLs, there is significant structural variability between different members of the family. There is a clear need for an improved understanding of their structure and mechanism. In particular structural information on enzyme-substrate complexes is required, even if only on substrate analogues or very poor substrates. In view of the key mechanistic roles of water molecules and of proton transfers, studies by neutron crystallography would be particularly valuable. In the light of the structural diversity among MBLs, a deeper understanding of their catalytic mechanism may be the most promising route to the development of clinically useful broad spectrum inhibitors.

Notes and References
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‡ Families of closely-related MBLs are denoted by three letters, followed by a number to denote an individual enzyme, e.g., NDM-1
§ The N220G mutant gave higher quality crystals than the wild-type, and showed a only very localised conformational difference.

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