Metallo-β-lactamases: Novel Weaponry for Antibiotic Resistance in Bacteria

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Received January 4, 2006

ABSTRACT
Metallo-β-lactamases are broad-spectrum zinc enzymes, able to inactivate most clinically useful β-lactam antibiotics. Their structural and functional diversity has thus far limited the understanding of their catalytic mechanism, therefore thwarting the rational design of a common inhibitor. On the basis of the recent availability of structures of enzyme−product complexes and novel mechanistic studies, here, we attempt to find minimal common elements in different members of this family. In contrast with other metalloenzymes, most of the substrate binding and catalytic power resides in the adequate positioning of one or two Zn^{II} ions in the active site, empowered by an unusual flexibility.

Introduction

β-Lactam antibiotics remain the most useful chemotherapeutic agents in the fight against bacterial infections.[1] Despite much progress in antibiotic design throughout 6 decades since the introduction of penicillin, resistance to β-lactams is now a serious clinical problem, particularly in postsurgery, nosocomial infections and immunosuppressed patients. The most prevalent resistance mechanism is the expression of enzymes tailored to hydrolyze these antibiotics, namely, β-lactamases.

Most β-lactamases possess an essential Ser residue in their active site. These enzymes have, on the basis of amino acid sequence, been grouped into three classes (A, C, and D).[1] Class B enzymes, instead, require 1 or 2 Zn^{II} ions bound to their active sites for activity and are collectively termed metallo-β-lactamases (MβLs). Despite a common fold and limited sequence homology, they have been divided into three different subclasses (B1, B2, and B3).[2] Subclass B1 is the largest family and contains the best studied MβLs, whose genes may be encoded either chromosomally or on mobile genetic elements that enable them to disseminate among pan-resistant Gram-negative pathogens such as *Pseudomonas aeruginosa*.[3]

MβLs display an unusually broad substrate profile, being able to inactivate all bicyclic β-lactam antibiotics. The exception is subclass B2, whose known members are exclusive carbapenemases.[3] This breadth and variability in the substrate spectrum, along with structural variation, have prevented ready generalizations regarding substrate binding and catalysis.

Recent reviews on MβLs have focused on their structure, inhibitor design, genetics of dissemination, and clinical significance.[3−5] Despite these efforts, the MβL mechanism remains substantially unresolved. Here, we attempt to summarize the present state of knowledge of MβL enzymology and, in doing so, highlight how these enzymes are distinguished from other metallohydrolases and to describe and challenge current mechanistic proposals.

Structure of Metallo-β-Lactamases

At the time of writing, crystal structures have been deposited for nine MβLs, including at least one representative from each of the three subclasses.[4] These and other structures make it clear that MβLs are prototypical members of a large and ancient metalloenzyme superfamily that includes a wide variety of hydrolases.[6] The common topology (Figure 1) is an $\alpha\beta/\beta\alpha$ sandwich fold, wherein the structural similarity between the two $\alpha\beta$ halves of the protein suggests that MβLs may well have arisen from a gene-duplication event.[7] The utility of this scaffold as a compact catalytic center upon which alternative specificities can be grafted with relative ease is highlighted by an elegant recent study, wherein a functional MβL was derived from the related human enzyme glyoxalase II.[69]

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10.1021/ar0400241 CCC: $33.50
Published on Web 07/14/2006

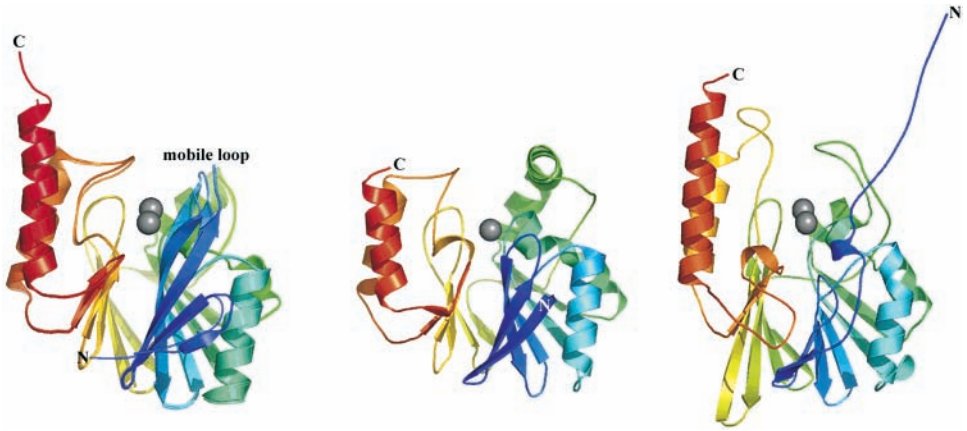

FIGURE 1. Representative metallo-β-lactamase structures: *B. fragilis* CcrA (B1, left) *A. hydrophila* CphA (B2, center), and *S. maltophilia* L1 (B3, right). The protein main chains are color-ramped from N (blue) to C (red) termini. Zinc ions are rendered as gray spheres. The left panel shows the superposition of the CcrA structure (PDB accession 1ZNB)[9] over the biphenyl tetrazole inhibitor complex (PDB accesssion 1A8T,[39] rendered semi-transparent). Note the movement of the unstructured active-site loop (unligated structure) over the active-site groove (inhibitor complex). Figures 1−3 were created with Pymol (www.pymol.org).

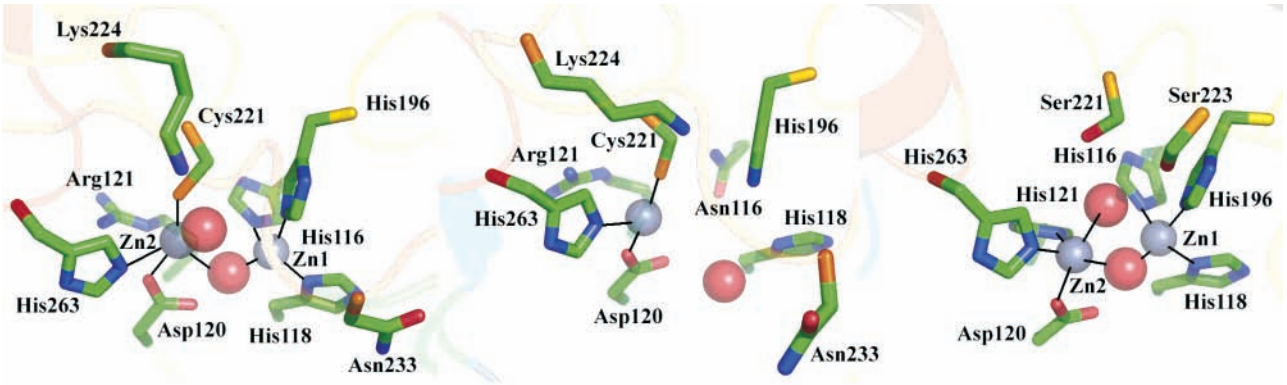

FIGURE 2. Metallo-β-lactamase active sites: *B. cereus* BcII (B1, left), *A. hydrophila* CphA (B2, center), and *S. maltophilia* L1 (B3, right). Zinc ions are rendered as gray spheres, and water molecules are rendered as red spheres. Coordination bonds are shown as solid lines.

The active site (Figure 2) is situated within a shallow groove formed by the interface of the two $\alpha\beta$ domains. Although the initial structure of *Bacillus cereus* BcII showed one zinc ion bound,[7] subsequent structures of B1 and B3 enzymes revealed a dinuclear metal center containing tetrahedral (Zn1) and trigonal bipyramidally (Zn2) coordinated zinc ions.[8] Each metal site includes elements from both halves of the protein and from a conserved H_{116}-$XH_{118}XD_{120}$ motif that characterizes the entire superfamily. In native (uncomplexed) structures, the zinc ions are 3.5−3.7 Å apart, with a "bridging" solvent molecule positioned asymmetrically with respect to the metal center and lying closer (1.9−2.1 Å) to Zn1 than to Zn2 (2.1−3.1 Å). The bridging geometry and short bond lengths associated with this molecule suggest it to be a hydroxide, a notion that is experimentally supported by the absence of acid pK_a values in the pH dependence of k_{cat} for dinuclear MβLs.[9] While there is close resemblance between the subclass B1 and B3 Zn1 sites, replacement of the B1 Zn2 ligand Cys221 by His121 in B3 enzymes affects the geometry of this site such that the coordination shell is rotated by 80° from its position in subclass B1 (Figure 2).[10] The mechanistic consequences of this alteration, which will affect the location of free coordination positions at Zn2, remain to be elucidated. Interestingly, other members of the MβL superfamily with different hydrolytic activities have Zn2 sites with a B3-like geometry[6] but possess at least one metal ligand bridging the two zinc ions (see below).

Several factors, including the comparatively large Zn^{II}−Zn^{II} separation and the asymmetric position of the bridging hydroxide, distinguish the MβL active site from other dizinc centers.[11] The presence of cysteine in a cocatalytic site is uncommon, although a metal-bridging Cys is observed in D-aminoacylase.[12] MβLs also differ from all of the above in that they lack a metal-bridging protein ligand (usually a carboxylate).

Various B1 MβLs have also been isolated with a single Zn^{II} ion bound to the Zn1 site.[7,68] This has been attributed to distinctly different affinities of the various enzymes for a second Zn^{II} ion. QM calculations suggest that, in the absence of Zn2, the Asp120−Cys221−His263 triad forms a hydrogen-bond network that steers the attacking hydroxide, replacing the role of Zn2 in dinuclear sites.[13−15] However, spectroscopic studies of mono-Zn^{II} BcII suggest that, at equimolar Zn^{II}/BcII, the metal ion is delocalized between the two binding sites,[16] while available high-resolution structures of mono-Zn^{II} B1 enzymes all show Cys221 to be oxidized. Consequently, the metal-ion occupancy and physiologically relevant form of mono-Zn^{II} BcII remains a matter of debate. Current efforts are being

made to generate mononuclear forms of L1 and CcrA to better evaluate the roles of each metal ion in catalysis and binding.

The recent crystal structure of *Aeromonas hydrophila* CphA reveals a different arrangement in subclass B2.[17] The structure shows a single tetrahedral zinc ion in the B1 Zn2 site (Asp120, Cys221, His263, and a carbonate ion), in agreement with spectroscopic studies.[18,19] Nonoccupancy of the Zn1 site can be attributed to the replacement of His116 (conserved in B1 and B3 enzymes) by Asn. The weaker-binding second Zn^{II} ion is inhibitory,[20] indicating that this enzyme is active in the mono-Zn^{II} form. The location of the inhibitory site remains unclear: although the Asn116His substitution increases its affinity,[21] suggesting it to be the B1 Zn1 site, spectroscopic evidence points to an alternative location.[19] Interestingly, the subclass B3 enzyme GOB-1 from *Elizabethkingia meningoseptica* displays a similar mutation (His116Gln), which may also lead to the elimination of the Zn1 site.[22]

The integrity of the metal center in all MβLs is maintained by a second shell that orients and polarizes the histidine ligands. Hydrogen-bond interactions with metal ligand backbone atoms have proven to be relevant in expanding the substrate spectrum in B1 MβLs, as shown by rational mutagenesis and directed evolution experiments.[23–25] It still remains to correlate these effects upon activity with possible changes in the MβL structure, dynamics, and/or stability.

Metal Content of MβLs in Vivo

One of the most controversial areas of research on MβLs is the issue of metal content of the enzymes in vivo. Wommer et al. suggested that MβLs are metal-free in vivo and bind 1 Zn^{II} only in the presence of the substrate.[26] This prediction was based on in vitro, thermodynamic binding studies where overexpressed, purified enzymes were made metal-free by dialyzes versus chelators, and the apoenzymes were titrated with Zn^{II}. This result has been challenged by Periyannan et al., who showed that L1 does not fold properly in vivo in the absence of Zn^{II}, suggesting that physiological metal incorporation into L1 is under kinetic rather than thermodynamic control.[27] It is unclear if physiological metalation of the other MβLs is under thermodynamic or kinetic control. This information is crucial to orient inhibitor design efforts.

Substrate Recognition by MβLs

Despite much recent progress, molecular recognition by MβLs remains incompletely understood. Nevertheless, accumulating evidence emphasizes the importance of the metal ion(s) to interactions with substrate. The most compelling evidence of the importance of the metal center to substrate binding by MβLs is provided by the observation that a properly folded apo-BcII is incapable of binding β-lactams.[28]

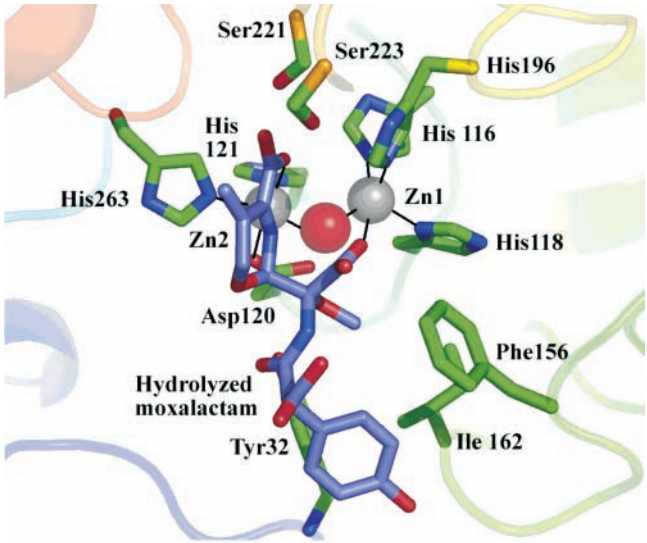

FIGURE 3. Structure of *S. maltophilia* L1 complexed with hydrolyzed moxalactam. Zinc ions are rendered as gray spheres, and water molecules are rendered as red spheres. Atom colorings are the same as the standard, except moxalactam carbon atoms (magenta).

Although a crystal structure for a MβL in complex with unhydrolyzed substrate remains elusive, the growing availability of MβL and inhibitor complex structures has revealed some common features.[4] Docking of substrates into the active sites of the di-Zn^{II} B1 and B3 enzymes has been guided by the assumed interaction between Zn1 and the β-lactam carbonyl oxygen that polarizes the carbonyl bond and renders the carbon susceptible to nucleophilic attack by the "bridging" hydroxide.[9,10] Residues Asn233 (B1) or Tyr228 (B3) may further assist this polarization, even if mutagenesis data indicate that this may not be true for all substrates.[29–31] The carboxylate moiety present in all bicyclic β-lactams, whose importance to tight binding is well-established,[32–34] is then positioned variously to contact either or both of the conserved Lys224 (subclass B1) and Zn2 and, in some cases, to displace the "apical" Zn2-bound water. The carboxylate/zinc interaction appears to be the main contributor to substrate binding by dinuclear model compounds.[35]

These conclusions are largely supported by the recent availability of crystal structures for two enzyme−product complexes: subclass B3 L1 enzyme with hydrolyzed moxalactam (Figure 3)[36] and subclass B2 CphA enzyme with hydrolyzed and rearranged biapenem.[17] The primary interactions in the dinuclear L1 enzyme involve (i) the substrate carboxylate displacing the "apical" Zn2-bound water molecule to occupy a pocket formed by the two serine residues Ser221 and Ser223 as a functional equivalent of Lys224 (B1 enzymes), (ii) the imine nitrogen of the 3′ exo-methylene hydrolysis product making direct contact with Zn2, (iii) the product carboxylate resulting from β-lactam cleavage acting as a Zn1 ligand, and (iv) the 7β-[(4-hydroxyphenyl)malonyl]-amino substituent contacting a hydrophobic patch. Trp39 may also contribute to the binding of substrates with bulkier substituents.[37] The main features (i and iii) of this mode of binding are thus consistent with many extant models for substrate binding

in subclass B1,[38] suggesting functional roles for two metal ions in substrate binding and catalysis.

The complex between CphA and hydrolyzed biapenem shows some common features with the previous picture: [17] the carboxylate and the nitrogen are within bonding distance of the metal ion (Zn2). The different orientation of the product can be attributed to loop topology at the active site, in which the extended interdomain helix α3 makes carbapenem-specific interactions with biapenem, to the absence of Zn1 and/or to the particular rearrangement experienced by biapenem upon hydrolysis. Nevertheless, the role of Zn2 in substrate binding is coincident with that observed for B3 and inferred for B1 enzymes.

Several investigations have also highlighted the importance of flexible loops that abut the B1 active site to the binding and/or hydrolysis of substrates by Mβ Ls. Concha et al. suggested that residues 60−66 of CcrA might form a flexible flap that could adjust to accommodate alternatively substituted substrates (Figure 1).[9] This view is vindicated by a comparison of the various enzyme−inhibitor structures[39,40] and by solution NMR[41] and molecular dynamics (MD) studies[42] that point to a repositioning and reduction in the flexibility of this region upon inhibitor binding. Mutagenesis data show the importance of this region and of aromatic residues within it to vary significantly with the substrate but indicate that it can serve as a hydrophobic cap to optimize the binding of a diverse range of substrates.[43] While the topology of active-site loops differs between B1 and B3 enzymes, the movement of the loop in L1 occurs at a rate that is kinetically competent.[37]

Far less is known about the binding of substrates by mononuclear B1 and B3 Mβ Ls. Models of substrate binding to BcII result in similar relative orientations of the β-lactam ring and the attacking nucleophile in the mono- and di-Zn^{II} forms.[44] A comprehensive mutagenesis study of the importance of individual residues to substrate binding in BcII is yet to be reported; interestingly, similar effects on K_M are observed for His mutations in both metal sites.[45]

These results collectively suggest that substrate binding to Mβ Ls is governed by a relatively small number of key determinants. The broad substrate specificity of Mβ Ls thereby arises in large part from the involvement of the metal ions in productive interactions with functional groups that are common to all substrates. To a lesser extent, additional protein motifs, such as the conserved residues and the flexible loops, may serve either to stabilize and/or polarize the substrate carbonyl or to optimize additional interactions while retaining the malleability that is required to accommodate the large array of different substrates.

Mechanistic Studies on Mβ Ls

β-Lactam hydrolysis involves two steps: a nucleophilic attack on the carbonyl group and C−N bond cleavage. The second step is generally triggered upon protonation of the bridging nitrogen. The bicyclic shape of β-lactams requires that the nucleophilic attack and nitrogen protonation take place on the same face of the antibiotic.[46] The attacking nucleophile in mono- and dinuclear B1 and B3 Mβ Ls is most likely the Zn1-bound hydroxide. The absence of covalent intermediates rules out the invariant Asp120, as an alternative possible nucleophile.[46,47] B2 Mβ Ls, lacking Zn1, should follow a different mechanism (see below).

Dinuclear Mβ Ls. The best understood dinuclear Mβ Ls are CcrA, IMP-1, and L1. Most studies have utilized nitrocefin, a chromophoric cephalosporin that, while not of therapeutic utility, undergoes intense spectroscopic changes upon hydrolysis that make it a useful mechanistic probe. Stopped-flow kinetic studies of nitrocefin hydrolysis by L1 and CcrA revealed the accumulation of a ring-opened, anionic intermediate.[48,49] This species has been observed in nitrocefin hydrolysis by a di-Zn^{II} model complex,[35] and its existence is supported by computational studies.[38] When these data are taken together, they indicate that Zn2 stabilizes a negative charge on the nitrogen atom after C−N bond cleavage. Rapid-freeze quench electron paramagnetic resonance (EPR) studies on Co^{II}-substituted L1 demonstrate that changes in the geometry of Co2 occur when this intermediate is formed and that similar changes accompany hydrolysis of other β-lactams.[50] However, the degree of accumulation of this intermediate varies markedly between enzymes and depends upon the protein environment. Nitrocefin differs from the majority of therapeutic β-lactams in the possession of an extended π-conjugated system that contributes to the stabilization of an anionic nitrogen. Studies of a wider range of substrates have led Spencer and co-workers to suggest that, in most cases, the amide bond is still intact in the populated enzyme-bound species.[51] Nitrogen protonation and C−N bond cleavage may not be distinguishable as two separate steps with other substrates.

A proposed reaction mechanism for dinuclear Mβ Ls that accounts for all previous work is shown in Figure 4. After a rapid-equilibrium binding of the substrate to the enzyme,[51] the bridging hydroxide becomes terminal as the β-lactam carbonyl interacts with Zn1.[52,53] Zn1 and other residues polarize the β-lactam carbonyl, rendering it susceptible to nucleophilic attack.[38] Deprotonated Asp120 orients the hydroxide to attack the β-lactam carbonyl carbon, generating a tetrahedral species[38,53−55] that, in contrast to the serine β-lactamase-catalyzed reaction, does not seem to accumulate.

This species collapses into a nitrogen−anionic intermediate that yields the product upon protonation.[47] This intermediate was postulated to be the species detected in stopped-flow UV−vis studies of nitrocefin hydrolysis.[56] The breakdown of either the tetrahedral or the nitrogen−anionic intermediate may be rate-limiting, depending upon the substrate and the identity of the amino acids in the active site.[29,30,51,57] The crystal structure of the L1-hydrolyzed moxalactam complex supports a role for Zn2 in stabilizing the transient development of a negative charge on the bridging nitrogen.[36] Asp120 and a water molecule have been suggested as possible proton donors in the rate-determining step, although this issue is still a

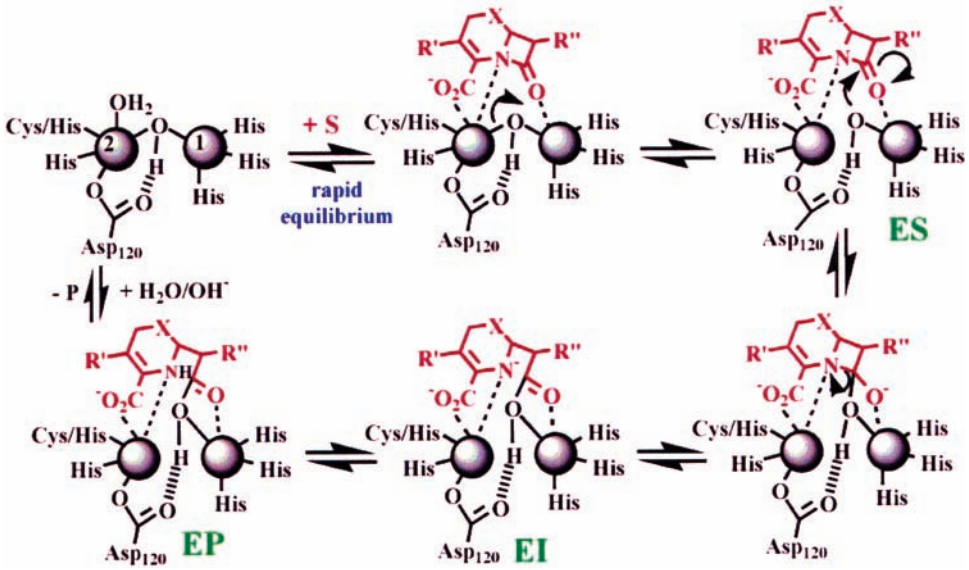

FIGURE 4. Proposed mechanism for cephalosporin hydrolysis by di-Zn^{II} B1 lactamases.

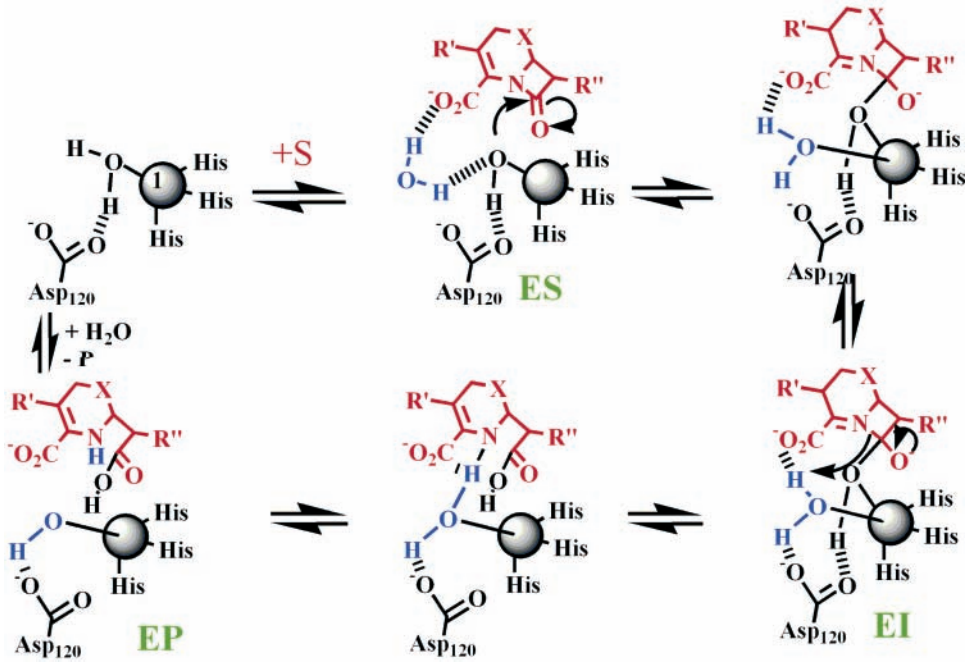

FIGURE 5. Proposed mechanism for cephalosporin hydrolysis by mono-Zn^{II} B1 lactamases.

matter of debate. Mutagenesis studies of Asp120 reveal that this residue is essential in CcrA, L1, IMP-1, and BcII.[29,45,55,58] Because Asp120 is predicted to be deprotonated in the resting enzyme,[52] it may be involved in the proton-transfer step by steering an incoming water molecule (Figure 4).[55]

The lack of evidence for a long-lived tetrahedral intermediate is consistent with the absence of a well-defined oxyanion hole in MβLs. Hence, any strategy aimed to design inhibitors based on transition-state analogues should differ from the one employed in serine–lactamases or proteases. It is also possible that cleavage operates by a concerted mechanism, wherein nucleophilic attack and amide bond cleavage (and in some cases, nitrogen protonation) occur simultaneously and tetrahedral oxyanion intermediates do not accumulate.

Mononuclear B1 MβLs. Despite extensive kinetic, structural, and computational studies on BcII,[8,13,43,46,59,60] there is, possibly due to the activity of both the mono- and dinuclear enzymes, no consensus on the reaction mechanism. Mono-Zn^{II} BcII has been proposed to be the physiologically relevant form.[26,61,62] Early mechanistic studies on BcII were performed in conditions of excess metal ion, in which the enzyme is predominantly dinuclear, but were interpreted in terms of a mono-Zn^{II} active site.[46,63,64]

A proposed mechanism for mono-Zn^{II} BcII is shown in Figure 5. This mechanism incorporates most of the data available on this enzyme; however, there are details shown that remain controversial. The resting form of the enzyme is assumed to be the tetrahedral Zn1 site. The Zn^{II}-bound water/OH^- is hydrogen-bonded to deprotonated

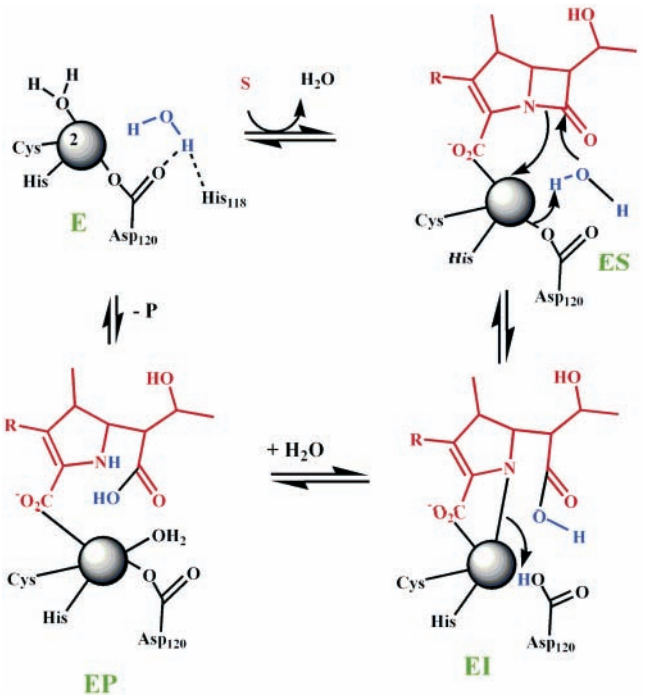

FIGURE 6. Proposed mechanism for carbapenem hydrolysis by mono-Zn^{II} B2 lactamases.

Asp120,[14,15,54,65] His263, and Cys221. Asp120 is oriented by Arg121 via two hydrogen bonds.

Both kinetic studies of the pH dependence of β-lactam hydrolysis[46] and crystallographic results[15] support the hypothesis that the pK_a of the Zn^{II}-bound water is 5.6. This protonation would be accompanied by partial or total dissociation of the Zn^{II} ion. As in dinuclear MβLs (Figure 4), this hydroxide serves as the reaction nucleophile, being oriented by Asp120.[13,62] During the breakdown of the tetrahedral intermediate, proton transfer to the β-lactam nitrogen is required. The source of this proton is not clear; however, His263,[59,60] Asp120,[46] and a water molecule[13] have been implicated as potential proton donors. As recently predicted with L1,[55] Asp120 may play a role in orienting water for proton donation (Figure 5). Mono-Zn^{II} BcII is not thought to stabilize anionic intermediates; however, controversy exists over whether the reaction of dinuclear BcII involves such species.[28,43,66]

The first mechanistic studies on BcII were reported in the mid-1980s. Using stopped-flow/rapid-scanning UV–vis kinetic and cryoenzymology studies, Bicknell et al. showed that Co^{II} BcII utilizes a branched kinetic pathway and several spectroscopically active intermediates were identified.[63,64] More work in this direction is needed.

Mononuclear B2 MβLs. Despite the lack of mechanistic studies in subclass B2 enzymes, the crystal structure of CphA complexed to hydrolyzed biapenem has inspired a catalytic mechanism in which the attacking nucleophile is a water molecule activated by His118 and/or Asp120, rather than a metal-bound water/hydroxyl (Figure 6).[17,67] The Zn^{II} ion (in the Zn2 site) would promote C–N bond cleavage by coordination to the bridging nitrogen atom.[67] Asp120 and a water molecule have been proposed as proton donors during catalysis, but mutagenesis and mechanistic studies are needed to validate these hypotheses.

Zn2: A Common Feature for all MβLs? The involvement of Zn2 in the B2 mechanism (species EI in Figure 6)[67] is equivalent to that indicated for species EI in the dinuclear B1 and B3 enzymes (Figure 4): Zn2 binds the nitrogen atom after C–N bond cleavage, as well as the carboxylate moiety present in all β-lactam substrates. The N–Zn2 interaction is supported by spectroscopic (nitrocefin hydrolysis by CcrA[48] and L1[49]) and crystallographic evidence (biapenem-CphA[17] and moxalactam-L1[36]) in all MβLs containing a Zn^{II} ion in the Zn2 site. Thus, with the possible exception of the elusive mono-Zn B1 enzymes, stabilization of the transition state for C–N bond cleavage by a Zn^{II} ion represents the only common mechanistic feature in all MβLs. This hypothesis could provide a starting point for designing a transition-state analogue-based inhibitor for all MβLs.

Perspectives and Future Directions

The ultimate goal in studying MβLs is to design and prepare clinically useful inhibitors that can be given in combination with existing β-lactams as a treatment for antibiotic-resistant bacterial infections. Preferably, this inhibitor would be effective against all MβLs. Despite almost 40 years of effort and several hundred research papers, there are still many unanswered questions that are needed to better guide rational inhibitor design.

The recent availability of structures for MβLs from all subclasses and the trapping of intermediates and products have provided clues for identifying common structural and mechanistic elements. However, many mechanistic details remain unresolved, especially in the B2 enzymes. Strategies to yield a more complete picture include the use of rapid-freeze quench spectroscopic studies and X-ray crystallographic studies with slow substrates or substrate analogues.

The lack of a consensus about how many metal ions are bound to B1 and B3 MβLs under physiological conditions and their role in catalysis remains a central issue to define a common inhibitory strategy.[69] Directed evolution could provide one means to test the in vivo metal-ion requirement for MβLs and to anticipate natural mutations.[25]

Among the various strategies developed by bacteria to survive β-lactam chemotherapy, MβLs undoubtedly arise as a growing resistance mechanism. Developing effective countermeasures to this growing threat will demand a huge effort from enzymologists, clinical microbiologists, structural biologists, and medicinal chemists in the coming years.

A. J. V. thanks ANPCyT and HHMI for financial support. A. J. V. is a staff member from CONICET. M. W. C. and J. S. thank the National Institutes of Health (GM40052) and the Beit Memorial Fellowships for Medical Research and the U.K. BBSRC for financial support, respectively. We apologize to our colleagues whose work

could not be discussed here because of space limitations. We thank all of our co-workers for their scientific input into the MβL projects in our labs.

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AR0400241