



University of HUDDERSFIELD

University of Huddersfield Repository

Page, Michael I. and Badarau, Adriana

The Mechanisms of Catalysis by Metallo β -Lactamases

Original Citation

Page, Michael I. and Badarau, Adriana (2008) The Mechanisms of Catalysis by Metallo β -Lactamases. *Bioinorganic Chemistry and Applications*, 2008. pp. 1-14. ISSN 1565-3633

This version is available at <http://eprints.hud.ac.uk/id/eprint/3380/>

The University Repository is a digital collection of the research output of the University, available on Open Access. Copyright and Moral Rights for the items on this site are retained by the individual author and/or other copyright owners. Users may access full items free of charge; copies of full text items generally can be reproduced, displayed or performed and given to third parties in any format or medium for personal research or study, educational or not-for-profit purposes without prior permission or charge, provided:

- The authors, title and full bibliographic details is credited in any copy;
- A hyperlink and/or URL is included for the original metadata page; and
- The content is not changed in any way.

For more information, including our policy and submission procedure, please contact the Repository Team at: E.mailbox@hud.ac.uk.

<http://eprints.hud.ac.uk/>

Review Article

The Mechanisms of Catalysis by Metallo β -Lactamases

Michael I. Page and Adriana Badarau

Department of Chemical and Biological Sciences, University of Huddersfield, Huddersfield HD1 3DH, UK

Correspondence should be addressed to Michael I. Page, m.i.page@hud.ac.uk

Received 5 November 2007; Revised 29 January 2008; Accepted 26 February 2008

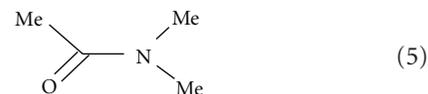
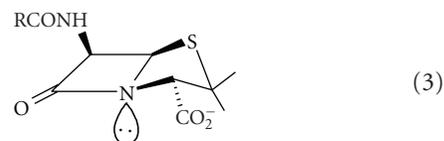
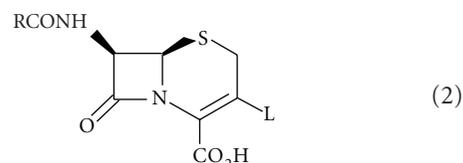
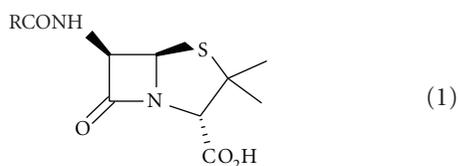
Recommended by Govindasamy Mugesh

Class B β -lactamases or metallo- β -lactamases (MBLs) require zinc ions to catalyse the hydrolysis of β -lactam antibiotics such as penicillins, cephalosporins, carbapenems, and cephamycins. There are no clinically useful inhibitors against MBLs which are responsible for the resistance of some bacteria to antibiotics. There are two metal-ion binding sites that have different zinc ligands but the exact roles of the metal-ion remain controversial, and distinguishing between their relative importance is complex. The metal-ion can act as a Lewis acid by co-ordination to the β -lactam carbonyl oxygen to facilitate nucleophilic attack and stabilise the negative charge developed on this oxygen in the tetrahedral intermediate anion. The metal-ion also lowers the pKa of the directly co-ordinated water molecule so that the metal-bound hydroxide ion is a better nucleophile than water and is used to attack the β -lactam carbonyl carbon. An intrinsic property of binuclear metallo hydrolytic enzymes that depend on a metal-bound water both as the attacking nucleophile and as a ligand for the second metal-ion is that this water molecule, which is consumed during hydrolysis of the substrate, has to be replaced to maintain the catalytic cycle. With MBL this is reflected in some unusual kinetic profiles.

Copyright © 2008 M. I. Page and A. Badarau. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. INTRODUCTION

All β -lactam antibiotics, such as penicillins (1) and cephalosporins (2), contain the four-membered β -lactam ring which occurs relatively rarely in nature, therefore it is not surprising that the biological activity of these compounds should be attributed to the expected enhanced chemical reactivity of the β -lactam ring [1]. It was suggested early that the antibiotic's activity was due to the inherent strain of the four-membered ring or to reduced amide-resonance. The nonplanar butterfly shape of the penicillin molecule (3) was expected to reduce amide-resonance and thus increase the susceptibility of the carbonyl group to nucleophilic attack, compared with normal, planar amides [1]. However, it was shown later that there is little evidence to confirm that the reactivity of β -lactams in penicillins and cephalosporins is due to an unusually strained or an amide-resonance inhibited β -lactam [2]. In fact, the rate of alkaline hydrolysis of the simple β -lactam (4) is only 3-fold greater than that of (5).

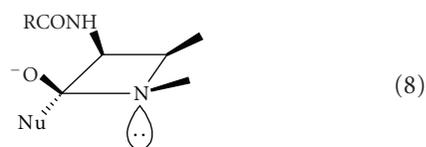
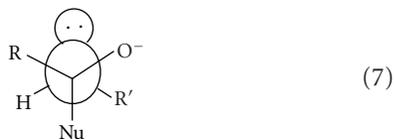
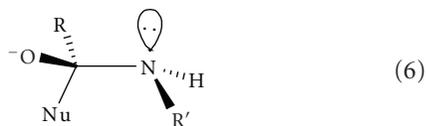


Most nucleophilic substitution reactions of β -lactams involve initial covalent bond formation between the carbonyl

carbon the of β -lactam and the attacking nucleophile followed by C–N bond fission of the β -lactam (Scheme 1).

This type of reaction is a two-step process and involves the reversible formation of a tetrahedral intermediate [1, 2]. Contrary to expectations, opening the four-membered ring is not a facile process [3] and, indeed, in many of these nucleophilic substitution reactions, the rate limiting step is not the first addition step but a subsequent one which may sometimes even be ring opening itself [1, 2, 4–8].

Another interesting difference between nucleophilic substitution in penicillins and peptides/amides is the preferred direction of attack and the geometry of the initially formed tetrahedral intermediate. It is usually assumed that nucleophilic attack on the carbonyl carbon of a planar peptide will generate a tetrahedral intermediate with the lone pair on nitrogen *anti* to the incoming nucleophile, (6), (7). Conversely, nucleophilic attack on the β -lactams of penicillins occurs from the least hindered α -face (*exo*) so that the β -lactam nitrogen lone pair is *syn* to the incoming nucleophile in the tetrahedral intermediate (8) [9]. This has obvious consequences for the placement of catalytic groups—particularly, if catalysis involves coordination to metal-ions [10].



2. β -LACTAMASES

It is more than sixty years since β -lactams were first introduced into clinical practice, but their therapeutic importance remains extremely high and they account for around half of total antibiotic prescriptions [11]. Given the rapid replication rates of bacteria against this selective pressure, it is not surprising that mechanisms of resistance continue to evolve and spread amongst pathogenic bacteria. Although there are several mechanisms of β -lactam resistance including modification of the target (transpeptidases), reduction in permeability, and efflux of the antibiotic, the major reason for resistance is antibiotic inactivation by the action of a variety of β -lactamases that are found in a large number of clinically important pathogens [12–14]. This number presently exceeds 500 and is growing (<http://www.lahey.org/studies/>).

β -Lactamases catalyse the hydrolysis of the β -lactam of penicillins (1) and cephalosporins (2) to give the ring opened and bacterially inert β -amino acid (Scheme 2) [15].

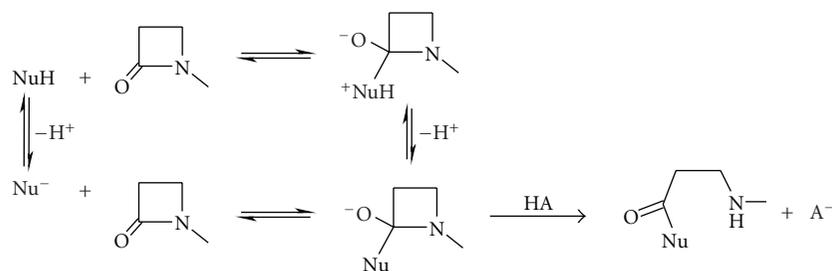
The main mechanistic division of β -lactamases is into serine enzymes and zinc enzymes [15]. The former have an active site serine residue and the catalytic mechanism involves the formation of an acyl-enzyme intermediate. The metallo enzymes appear to involve coordination of the substrate and intermediates to the active site metal-ion. On the basis of their amino acid sequences, the serine β -lactamases are subdivided into three classes: A, C, and D, whereas the class B β -lactamases consist of the zinc enzymes [15].

3. METALLO β -LACTAMASES

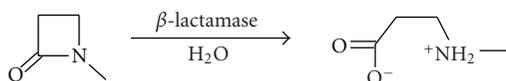
Class B β -lactamases or metallo- β -lactamases (MBLs) require zinc-ions to catalyse the hydrolysis of β -lactams and have no sequence or structural homology to the serine β -lactamases. They exhibit a broad spectrum substrate profile catalysing the hydrolysis of a wide range of β -lactam antibiotics including penicillins, cephalosporins, carbapenems, cephamycins, and even some mechanism-based inhibitors of class A β -lactamases [16]. The first metallo- β -lactamase to be discovered was produced by an innocuous strain of *Bacillus cereus*, but in the last 20 years, MBL-mediated resistance has appeared in several pathogenic strains and is being rapidly spread by horizontal transfer, involving both plasmid and integron-borne genetic elements [17]. MBLs represent a huge potential clinical threat to the β -lactam antibiotic therapy as presently there are no clinically useful inhibitors for them.

MBLs can be divided in three subclasses: subclass B1, B2, and B3 based on their amino acid sequences, substrate profile, and metal-ion requirement [18, 19]. Subclass B1 is the largest and contains several well-studied β -lactamases: BCII from *Bacillus cereus*, CcrA from *Bacteroides fragilis*, IMP-1, SPM-1 from *Pseudomonas aeruginosa*, and BlaB from *Cryseobacterium meningosepticum*. These enzymes efficiently catalyse the hydrolysis of a wide range of substrates such as penicillins, cephalosporins, and carbapenems. BCII catalyses the hydrolysis of penicillins at significantly higher rates than cephalosporins and carbapenems, but CcrA does not show this preference, although both enzymes exhibit lower K_m values for cephalosporins [20].

The most common representatives of subclass B2 are CphA from *Aeromonas hydrophila* and ImiS from *Aeromonas veronii*, which preferentially hydrolyze carbapenems, for example, imipenem and meropenem, but have poor activity against penicillins and cephalosporins [20, 21]. Finally, subclass B3 contains the only known tetrameric zinc β -lactamase, the L1 enzyme from *Stenotrophomonas maltophilia*, and the monomeric FEZ-1 from *Legionella gormanii*. Both enzymes hydrolyze a wide range of β -lactam antibiotics [22, 23] with L1 having higher catalytic rate constants (k_{cat}) for penicillins compared with FEZ-1, which shows higher k_{cat} values for cephalosporins. Generally, the K_m values are smaller for L1 than for FEZ-1. However, mutation of a methionine residue in L1, which is important



SCHEME 1



SCHEME 2

for the subunit interaction, gives a monomeric enzyme with significantly higher K_m values and, except for nitrocefin, smaller k_{cat} values compared with the wild-type tetramer [24].

3.1. The number of zinc-ions

A major problem with understanding the mechanism of MBLs is the number of zinc-ions required for catalysis, which has been addressed by studying MBLs under both equilibrium and kinetic conditions. The crystal structures of several MBLs have been determined by X-ray diffraction and they all show a similar $\alpha\beta/\beta\alpha$ sandwich fold, which was first seen with MBLs [25], but since recognised in other enzymes, such as glyoxalase II, aryl sulfatase, and cAMP phosphodiesterase, which have now become members of the MBL fold superfamily [26]. The active site of MBLs is situated at the bottom of a wide shallow groove between two β -sheets and has *two* potential zinc-ion binding sites at the active site, often referred to as sites 1 and 2 [27–29]. The zinc ligands in the two sites are not the same and are not fully conserved between the different MBLs. Table 1 shows the enzyme residues involved in zinc coordination in the two binding sites in the subclasses B1, B2, and B3 [26].

In the subclass B1, such as the *Bacillus cereus* enzyme BCII, the zinc in site 1 (the histidine site or His₃ site) is tetraordinated by the imidazole groups of three histidine residues (116, 118, and 196) and a water molecule, Wat₁. In site 2 (or the Cys site) the metal is pentacoordinated by His263, Asp120, Cys221, and one water molecule; the fifth ligand at site 2 is carbonate or water, often referred to as the apical water, or Wat₂ [27, 28, 30], although this is missing in structures with inhibitors bound [31]. The two metal-ions are relatively close to each other, but the distance between them varies from 3.4 to 4.4 Å in different structures of the BCII and CcrA enzymes. Several structures of the CcrA enzyme show a bridging water ligand between the two metals, which is thought to exist as a hydroxide-ion [32]. In a structure of BCII determined at pH 7.5 that contains two

zinc-ions there is also a similar bridging water molecule, but in structures of this enzyme at lower pH this solvent molecule is strongly associated to the zinc in site 1 [30].

The two conserved zinc binding sites in MBLs have different metal-ion affinities. For example, the BCII enzyme from *Bacillus cereus* has very different dissociation constants for the two metal binding sites. The first crystal structure, obtained at low pH [25], indicated only one zinc-ion bound to the histidine site, but equilibrium dialysis studies showed two binding events, with a dissociation constant of 0.3 μ M for the first zinc-ion (K_{mono}) and 3 μ M for the second zinc-ion (K_{bi}) [33]. Later metal binding studies by fluorescence spectroscopy using a chromophoric chelator reported a K_{mono} of 0.62 nM and a K_{bi} of 1.5 μ M [34]. As the conditions of the experiments were similar for the two studies, the large discrepancy in K_{mono} may be due to the fact that different strains of BCII enzyme from *Bacillus cereus* were used. The dissociation constants K_{mono} and K_{bi} have been determined from the steady state rates of hydrolysis of imipenem in the presence of EDTA as a metal-ion buffer at different zinc concentrations and K_{mono} was found to decrease significantly, from nM to pM, in presence of substrate, whereas K_{bi} decreased only by two fold [35]. This led to the suggestion that the monozinc enzyme is responsible for the catalytic activity under physiological conditions, where the concentration of the “free” Zn^{2+} is in the pM or even fM region. For most substrates, the reported catalytic activity of the monozinc BCII was about two fold lower than that of the binuclear enzyme. Conversely, the other class B1 enzyme, CcrA from *Bacteroides fragilis*, binds both zinc-ions very tightly [36]. Despite the very close similarity with BCII, CcrA has much higher affinity for the second zinc-ion, probably due to the fact that in CcrA, a cysteine residue replaces the positively charged arginine 121 found in BCII. However, replacing Arg 121 in BCII by a Cys shows no increase in the affinity for the second zinc-ion [37].

Early kinetic studies of CcrA led to the proposal that *both* the mono and the dinuclear forms of the enzyme were catalytically active, with slightly different activities, at physiological pH [38]. However, later studies showed that only the dinuclear species was active and that the previously observed “monozinc” CcrA was a mixture of the dizinc and the apo (metal free) enzyme [39]. Class B2 metallo β -lactamases are catalytically active with one bound zinc-ion and the binding of the second zinc-ion inhibits the enzyme noncompetitively, with a K_i of 50 μ M [40]. The dissociation

TABLE 1: The zinc ligands in class B β -lactamases.

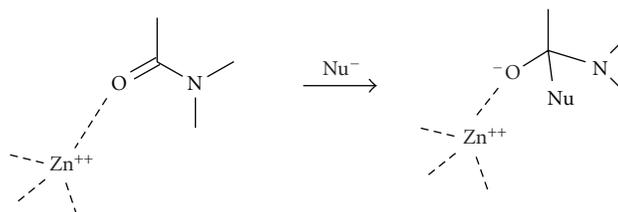
Subclass	Zn1 ligands				Zn2 ligands	
B1	His116	His118	His196	Asp120	Cys221	His263
B2	Asn116	His118	His196	Asp120	Cys221	His263
B3	His/Gln116	His118	His196	Asp120	His121	His263

constant of the first zinc-ion was found to be 7.0 and 1.2 pM in the absence and presence of substrate (imipenem), respectively [35]. Subclass B3 MBLs, L1, and FEZ-1 bind both metal-ions tightly, the dissociation constants are in the nM region, and are fully catalytically active in binuclear form [23, 41].

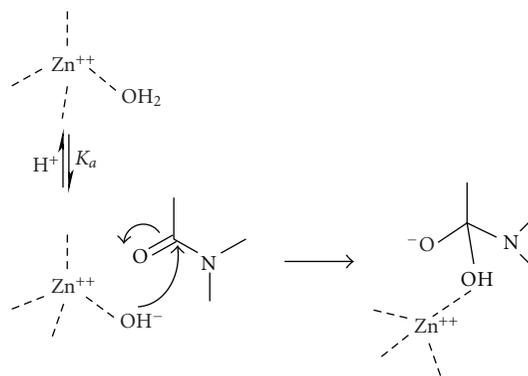
3.2. The catalytic role of zinc

Zinc is an essential trace element and the second most abundant transition metal found in living systems. Its role in catalysis is related to its ability to participate in tight, but readily exchangeable ligand binding and its exceptional flexibility of its coordination number and geometry [42]. In addition, zinc shows no redox properties and this facilitates its evolution in living systems without the risk of oxidative damage. Finally, its intermediate hard-soft behaviour allows it to bind a variety of atoms, as seen, for example, in the second binding site of class B1 MBLs which involve nitrogen, oxygen, and sulphur as ligands. The Lewis acidity, flexible geometry, and coordination number and the lack of redox properties make zinc an ideal metal cofactor for many enzymes. The small energy difference between 4, 5 or 6 coordination geometries and the rapid exchange of the kinetically labile zinc-bound water molecule are important features in all zinc hydrolases including MBLs.

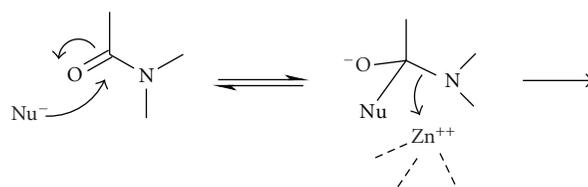
MBLs are a subclass of metallo-proteases and many mechanistic considerations are applicable to both groups. There are many potential mechanistic roles for the metal-ion in metallo-proteases and MBLs, although they may well vary from enzyme to enzyme [42, 43]. It is commonly suggested that the metal-ion acts as a Lewis acid by coordination to the peptide carbonyl oxygen giving a more electron deficient carbonyl carbon which facilitates nucleophilic attack. The metal-ion thus stabilises the negative charge developed on the carbonyl oxygen of the tetrahedral intermediate anion (Scheme 3). Many metallo-proteases have a water molecule directly coordinated to the metal-ion which may act as the nucleophile to attack the carbonyl carbon. Here, the role of the metal-ion is to lower the pK_a of the coordinated water so that the concentration of metal-bound hydroxide ion is increased relative to bulk solvent hydroxide-ion at neutral pH and, furthermore, although this coordinated hydroxide-ion is different in nature than the simple solvated ion, it is a better nucleophile than water (Scheme 4). These two steps shown in Schemes 3 and 4 relate to formation of the tetrahedral intermediate and although little attention is normally given to the mechanism of the breakdown of the tetrahedral intermediate, C–N bond fission is the most energetically difficult process in peptide hydrolysis. This



SCHEME 3



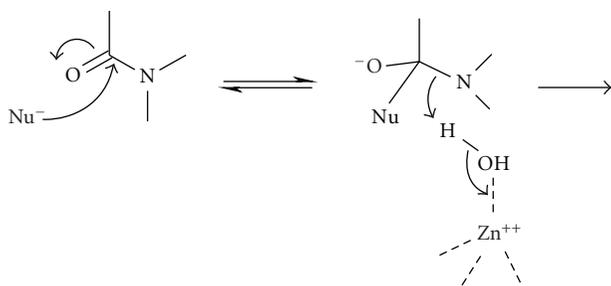
SCHEME 4



SCHEME 5

could be facilitated by direct coordination of the departing amine nitrogen to the metal-ion (Scheme 5) which is, in fact, the mechanism adopted for the simple zinc-ion-catalysed hydrolysis of penicillin [10]. Alternatively, a metal-bound water could act as a general acid catalyst protonating the amine nitrogen leaving group to facilitate C–N bond fission (Scheme 6). Despite intense mechanistic studies, the detailed roles of the metal-ion in metallo-proteases and MBLs remain controversial and distinguishing between the relative importance of the possible roles for zinc is complex.

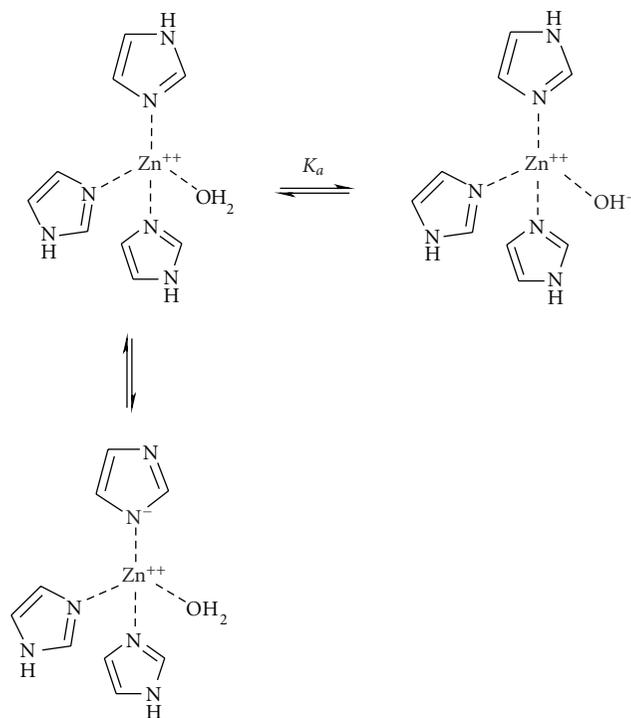
The effective positive charge on the zinc-ion depends on the number and nature of its ligands and for those with an



SCHEME 6

ionisable hydrogen, zinc lowers their pK_a and the ionised ligand obviously is better at neutralising the positive charge density on the metal. The pK_a of water is 15.7, but when bound to zinc in aqueous solution it is lowered to 9.5, but is there evolutionary pressure to lower the pK_a of the zinc-bound water in an enzyme even further? This could be achieved by replacing say a negatively charged carboxylate ligand by a neutral histidine. For example, in the opposite direction, changing the histidine bound to zinc in carbonic anhydrase to aspartate increases the pK_a of the zinc-bound water from 6.8 to ≥ 9.6 [45]. Whether it is better to have a higher or lower pK_a metal-bound water for a faster reaction and more efficient catalysis depends on the role of the zinc-ion. A low pK_a implies a more electron deficient metal-ion centre which would give a better Lewis acid to stabilise the negative charge developed on the oxyanion of the tetrahedral intermediate. Conversely, a high pK_a for the metal-bound water implies a weaker Lewis acid and so the zinc-ion will be less efficient at stabilising the tetrahedral intermediate. A low pK_a for the metal-bound water implies that the hydroxide-ion resulting from ionisation is more “tightly bound” and stabilised which, although it becomes the dominant species even at low pH, corresponds to a more weakly nucleophilic hydroxide-ion. For example, if the pK_a of the zinc-bound water is about 5 then the nucleophilicity of the metal bound hydroxide-ion is only similar to that of a carboxylate anion. If a major role of the metal-ion is to provide a better nucleophile than water then the net effect depends on the relative importance of concentration and the dependence of the rate upon nucleophilicity. If the pK_a is “too high”, metal-coordinated water will be the dominant species over the desired pH range but deprotonation will give a more nucleophilic metal-bound hydroxide. How reactivity changes with changing pK_a and pH will depend on the susceptibility of the rate of reaction to the basicity of the nucleophile—the hydroxide-ion bound to the metal—as indicated by the Bronsted β_{nuc} value.

Sometimes it is suggested that part of the enzyme mechanism involves general base catalysis to remove the proton from the zinc-bound water but this only becomes necessary if the pK_a is little changed from that of bulk water. A problem with the commonly accepted mechanism for metallo-proteases of rate-limiting deprotonation of zinc-bound water concerted with nucleophilic attack is the pK_a of this water. Coordination to zinc(II) generally lowers the

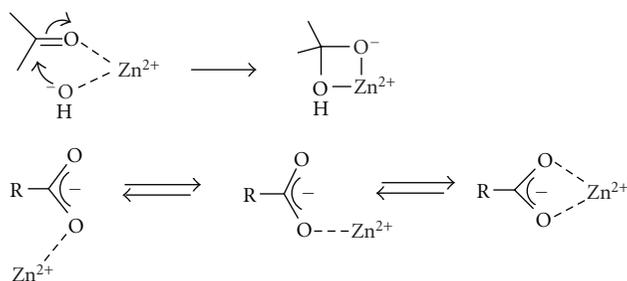


SCHEME 7

pK_a of water to 5–9 depending on the number and type of other ligands. The concentration of zinc-bound hydroxide ion is therefore quite high over the normal pH range studied. There is little or no *catalytic* advantage in having a general base to remove a proton in this pre-equilibrium step! Even if the pK_a of the zinc(II)-bound water is about 9 then 10% of the species already exists in the fully deprotonated form at pH 8 and 1% at pH 7. Presumably, the deprotonated form is a much better nucleophile than the species which is only partially deprotonated and there would be no catalytic advantage of the general base-catalysed mechanism.

In aqueous solution, zinc is coordinated to six water molecules and the pK_a of the zinc-bound water is 9.5. However, within a protein this pK_a can be changed significantly because of the environment and directly bonded ligands to the metal—for example, the zinc of *B. cereus* β -lactamase is coordinated to three protein ligands in the zinc1 site—His 116, His 118, and His 196 and a water molecule [30]. The reduced coordination number of four in the zinc1 site of β -lactamase reduces the pK_a to less than 6. In principle, one or more of the imidazole residues could be ionised, the pK_a for proton loss from imidazole is similar to that of water (about 14). However, NMR evidence suggests that the imidazole of the bound histidines are neutral [46, 47] and the most likely group to ionise is, therefore, the bound water (Scheme 7).

One way to investigate the role of the zinc in catalysis is to modify the pK_a of the zinc-bound water by changing the ligands or the metal-ion. If the activity of the resulting enzyme *increases* as the pK_a of the zinc-bound water ligand *decreases*, this would suggest that a high positive charge density on the metal-ion facilitates catalysis and that the



SCHEME 8

A similar intermediate has been observed during the class B3 L1 enzyme-catalysed hydrolysis of nitrocefin, and, as in the case of CcrA, its breakdown was rate-limiting [53]. However, stop-flow studies of the tryptophan fluorescence revealed that the mechanism of nitrocefin hydrolysis by binuclear metallo- β -lactamases may be unusual and cleavage of the β -lactam amide bond is the rate determining step for the breakdown of the majority of β -lactam substrates by the L1 enzyme [54].

In contrast to these results, no accumulation of an anionic intermediate could be detected in reactions catalysed by BCII and the rate of substrate depletion and product formation are similar [55]. However, for the BCII-catalysed hydrolysis under cryoenzymological conditions, a slightly red-shifted intermediate (440 nm) was observed, which was suggested to be a nitrocefin-like tetrahedral intermediate [56].

An unusual mechanism has been proposed for subclass B2 metallo- β -lactamase CphA from *Aeromonas hydrophila* based on the crystal structures of the free enzyme and of a reaction intermediate between the enzyme and the substrate meropenem (Scheme 13) [57]. Nucleophilic attack is performed by a water molecule not coordinated to zinc, but activated by general base catalysis by His118, followed by C–N bond fission which occurs prior to nitrogen protonation. This proposal uses zinc as a Lewis acid to facilitate C–N bond fission and to stabilise the leaving group—a mechanism proposed much earlier based on model studies [10]—whereas most mechanisms use the zinc-ion to act as a Lewis acid to stabilize oxyanion formation from the β -lactam carbonyl oxygen. Rearrangement of the proposed intermediate amine anion, whose negatively charged nitrogen is stabilised by an interaction with the zinc-ion, gives the bicyclic intermediate shown (Scheme 13), which is observed in the crystal structure, although this rearrangement may not occur in the active site of the enzyme. A solvent molecule replacing the water used in the nucleophilic attack protonates the nitrogen and releases the final product.

3.4. Mutation studies with MBLs

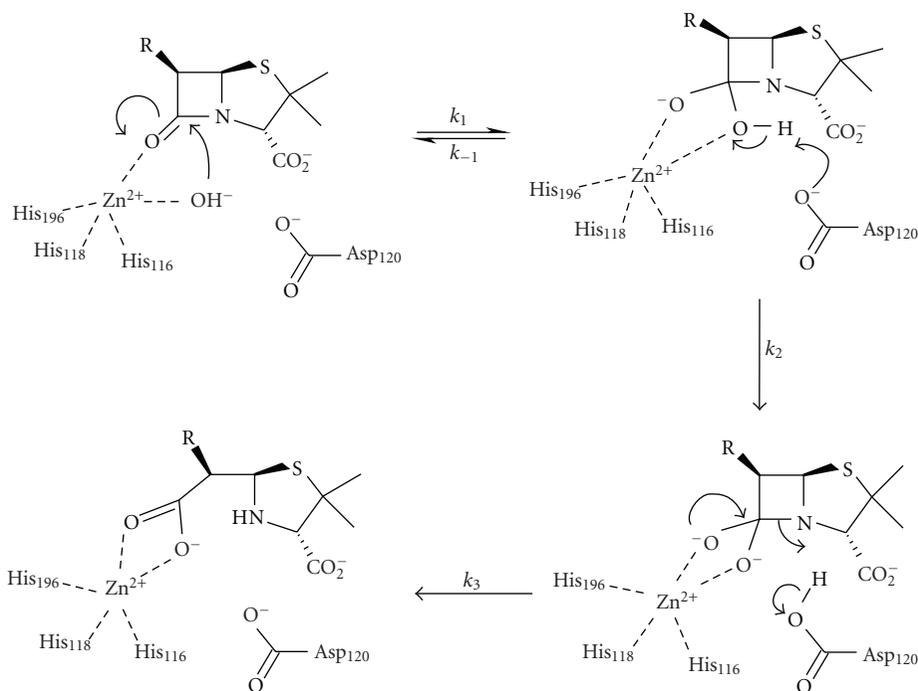
Many mutant enzymes from the three subclasses of MBLs have been produced and characterised. In general, the residues substituted have been the metal ligands, those providing the framework for orienting the metal ligands

or those thought to interact with the substrates. For BCII, replacing the histidine residues in the first binding site with serine resulted in decrease of catalytic activity, although the affinity for zinc was not significantly altered [58]. The decrease in activity appeared as due to an increase in K_m which was rationalised on the basis that, in absence of a His ligand, there is a higher degree of flexibility of the substrate inside the catalytic site.

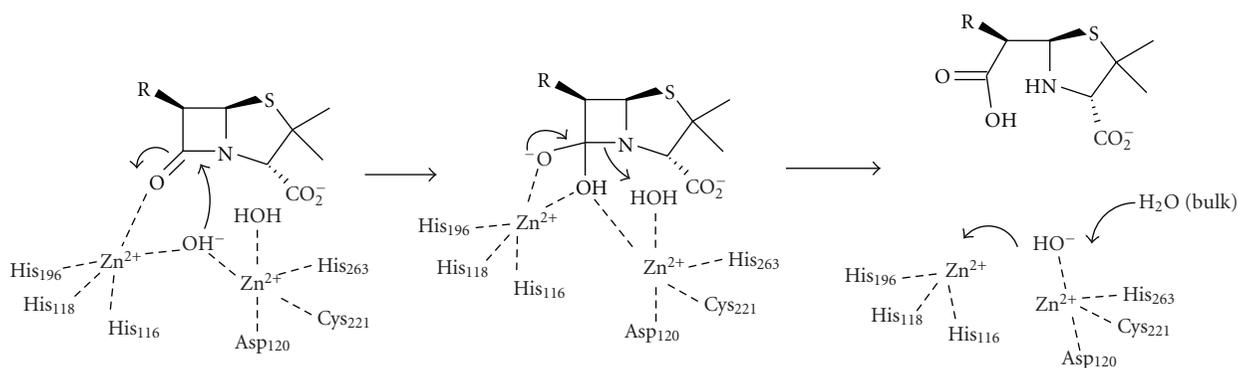
The replacement of the Cys221 residue with alanine or serine in the active sites of BCII [58], CcrA [59], and IMP1 [60] gives a drastic decrease in the rate of hydrolysis at low zinc concentrations, but the mutants are “reactivated” by the addition of excess zinc, at least in case of BCII and IMP1. It was proposed that the presence of Cys has a crucial importance for the catalytic activity of the monozinc enzyme but not for the dinuclear species [58].

Substituting the zinc 2 ligand Asp120 in BCII with Asn reduces the catalytic activity by more than 100 fold, but does not impair the binding of the second zinc-ion [58]. Although these results support the hypothesis that Asp120 plays an important role in catalysis, the significant catalytic activity of the mutants is inconsistent with Asp playing the role of a general base. The same amino acid substitution in subclass B3 MBL enzyme L1 led to an average 10 fold decrease in catalytic activity in the binuclear mutant [61]. It is interesting that for the hydrolysis of nitrocefin, this mutant shows an unusually large kinetic solvent isotope effect (KSIE) of 5.36 compared with the wild type (KSIE = 2.08) and other aspartate mutants: Asp120Cys and Asp120Ser (KSIE < 2). It is concluded that the Asp120 residue is essential for the catalytic activity in the orientation of the bridging group of the L1 enzyme, in the protonation step of breakdown of the tetrahedral intermediate, rather than in its formation involving nucleophilic attack, as previously suggested [62].

The mutation of the second shell ligand, Arg121Cys, in BCII gives an enzyme with a lower affinity for the second metal-ion compared to the wild type [58]. Moreover, the electronic spectrum of the dicobalt Arg121Cys BCII mutant is identical to that of the wild-type enzyme indicating that the mutation has not altered the metal coordination sphere. The catalytic activity of the mutant is only two fold lower than that of the wild type for both the mono- and dinuclear forms of the enzyme. The Cys104Arg mutation in CcrA led to a binuclear enzyme with significantly decreased activity and which, unlike the wild type, can have one bound metal-ion removed by a chelator [39]. Presteady state kinetics suggested a change in the rate limiting step in the hydrolysis of nitrocefin, from nitrogen protonation in the wild type, to C–N bond fission in the mutant, which showed a catalytic rate constant similar to that of the dizinc BCII enzyme. The monozinc form of the mutant showed a k_{cat} value similar to that of the monozinc BCII, for nitrocefin hydrolysis, with a further decrease of the rate of C–N bond fission compared with the dizinc mutant, but with a similar protonation rate. Based on these observations it was concluded that both the replacement of the arginine residue and the introduction of the second metal-ion are evolutionary tools for accelerating C–N bond fission in MBL catalysed hydrolysis of β -lactams. However, an important role in lowering the energy barrier



SCHEME 11



SCHEME 12

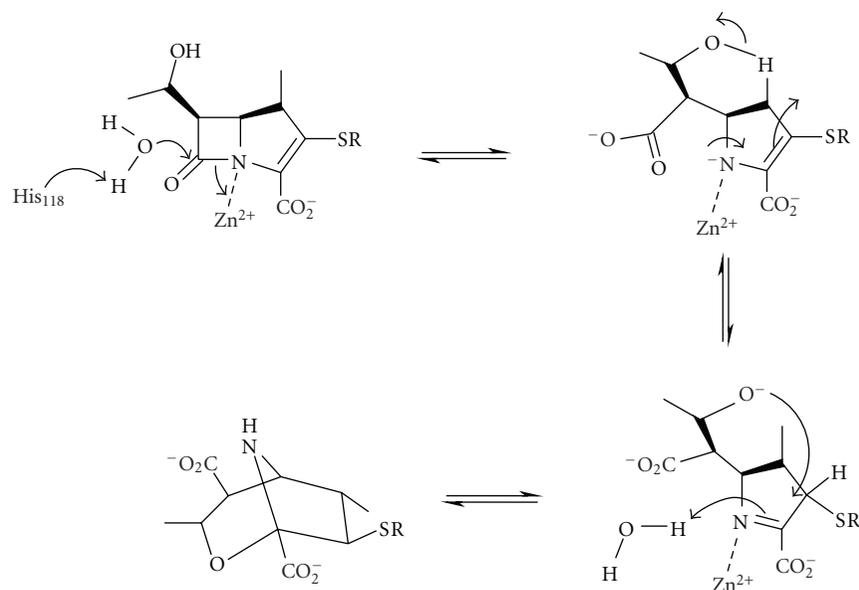
better substrates. Moreover, the activity of the Asn116His mutant increased with increasing metal-ion concentration for the hydrolysis of benzylpenicillin and cephaloridine, as opposed to imipenem, where addition of zinc to both the mutant and the wild type lead to noncompetitive inhibition. The Cys221Ser and Cys221Ala mutants were seriously impaired in their ability to bind the first zinc-ion and were nearly completely inactive indicating a major role for Cys221 in binding the catalytic metal-ion.

3.5. Metal ion substitution

The exchange of the spectroscopically silent zinc in zinc enzymes with probes, such as cobalt, copper, and cadmium, enables the study of the metal interactions in the enzyme active site with its ligands, substrates, and inhibitors of the metallo-enzyme using techniques such as electronic

spectroscopy, NMR, EPR, and perturbed angular correlation (PAC) spectroscopy. The zinc of metallo β -lactamases can be exchanged with cadmium, cobalt, and manganese to give catalytically active enzymes. The use of a combination of NMR and PAC spectroscopy to study cadmium binding to *B. cereus* MBL has revealed a rapid intramolecular exchange of the metal between the two sites in the monocadmium enzyme and negative cooperativity in metal binding [64]. The enzyme inhibitor (R)-thiomandelate induces a very strong positive cooperativity for binding the second cadmium cation [65].

The metal-ion environment of cobalt-substituted metallo β -lactamases has been studied by UV-vis spectroscopy, NMR, and ESR. The UV-vis spectra of cobalt-substituted class B1 metallo- β -lactamases CcrA from *Bacteroides fragilis* [36, 66] and BCII from *Bacillus cereus* [67, 68] show similar features with an intense S to Co(II) ligand to metal charge



SCHEME 13

transfer transition (LMCT) band at 340 and 348 nm and four characteristic d-d transition bands between 500 and 650 nm. The spectrum of Co-BCII in the visible region is also very similar to that of Co(II) carbonic anhydrase at alkaline pH [69], suggesting that the contributions from the His₃ metal centre dominate this part of the spectrum of Co substituted class B1 MBLs.

On titration of monozinc BCII with Co(II), the spectrum shows an LMCT band, but without the four ligand field bands which suggests that zinc binds to the His₃ site, while cobalt preferentially occupies the Cys site in the hetero-substituted CoZn-enzyme [67]. A similar discrimination between metals in occupying the two binding sites in BCII was noted for the CdZn-enzyme, where zinc also binds in the His₃ site and Cd to the Cys site [64]. Studies of cobalt binding to apo BCII, using HEPES as a buffer and no added sodium chloride, showed that increasing the ratio Co(II)/enzyme above one resulted in a decrease of the charge transfer band at 344 nm and the appearance of an additional charge transfer band at 383 nm. This has been explained by the binding of a second cobalt ion to the monocobalt BCII species and the existence of two different Co(II) LMCT band positions, one for the mononuclear (344 nm) and one for the binuclear enzyme (383 nm); this latter band disappears upon addition of sodium chloride, accompanied by an increase in absorbance at 344 nm, which led to the suggestion that chloride hinders the binding of a second cobalt ion [34]. More recent data supported by NMR and EPR evidence have shown that the band at 383 nm corresponds to a third, more weakly bound cobalt ion that perturbs the 344 nm signal (the Cys site), without affecting the spectral features of the histidine site [70].

Low-temperature EPR spectra of metallo- β -lactamases indicate the presence of high spin, but not coupled, Co(II) ions in a rhombically distorted pentacoordination sphere in

Co-substituted BCII [68], a penta/hexacoordination environment in Co-substituted CcrA enzyme [36], a tetra-coordination environment for the first cobalt ion, and penta/hexacoordination sphere for the second cobalt ion in Co-substituted ImiS [71]. For this latter enzyme, it has been shown by EPR that the second cobalt ion is magnetically isolated, suggesting a distance of more than 7 Å from the first bound cobalt ion. Furthermore, NMR titration of the monocobalt ImiS enzyme with Co(II) indicated that the second cobalt ion is not bound to histidine, as the newly observed resonances are not solvent exchangeable; these findings were taken to indicate that the Zn1 site has no catalytic or metal binding role in ImiS, that Zn2 site binds the metal-ion that is required for catalysis, and a remote, lower-affinity, metal binding site is responsible for the noncompetitive inhibition of the enzyme [72]. From the mechanistic point of view, it has been shown by time dependent UV-vis spectroscopy and fluorescence quenching, using nitrocefin as a substrate, that Co-substituted L1, a class B3 metallo- β -lactamase, probably utilises a reaction mechanism similar to that of the native zinc enzyme [54, 73]. Rapid-freeze-quench (RFQ) EPR shows that a short-lived intermediate is a metal-bound species, and the role of the metal-ion in catalysis is similar for nitrocefin, cephalothin, meropenem, and benzylpenicillin [73].

The kinetics and mechanism of hydrolysis of the *B. cereus* (BcII) metallo- β -lactamase substituted with various metal-ions have been investigated to determine the role of the active site metal-ion [74]. The pH and metal-ion dependence of k_{cat} and $k_{\text{cat}}/K_{\text{m}}$ for the cobalt-substituted BcII catalysed hydrolysis of cefoxitin, cephaloridine, and cephalixin indicate that an enzyme residue of apparent pK_{a} 6.3 is required in its deprotonated form for metal-ion binding and catalysis. The $k_{\text{cat}}/K_{\text{m}}$ for cefoxitin and cephalixin hydrolysis with cadmium-substituted BcII is

dependent on two ionising groups on the enzyme: one of pK_{a1} 8.7, required in its deprotonated form, and the other of pK_{a2} 9.3, required in its protonated form for activity. The identity of these residues was determined from the pH-dependence of the competitive inhibition constant, K_i , of the Cd BcII by L-captopril which showed that the pK_{a1} of 8.7 corresponds to the cadmium-bound water. For the manganese-substituted BcII, the pH-dependence of k_{cat}/K_m for the hydrolysis of β -lactam antibiotics similarly indicated the importance of two catalytic groups: one of pK_{a1} 8.5 which needs to be deprotonated and the other of pK_{a2} 9.4 which needs to be protonated for catalysis; the pK_{a1} was assigned to the manganese-bound water [74]. Interestingly, the metal-substituted enzymes have similar or higher catalytic activities compared with the native zinc enzyme, albeit at pHs above 7 and, for the Co enzyme, at all pHs (Figure 1). With cefoxitin, a very poor substrate for Zn BcII, both k_{cat} and k_{cat}/K_m increase with increasing pK_a of the metal-bound water, in the order $Zn < Co < Mn < Cd$. A higher pK_a for the metal-bound water for cadmium and manganese BCII leads to more reactive enzymes than the native zinc BcII, suggesting that the role of the metal-ion is predominantly to provide the nucleophilic hydroxide, rather than to act as a Lewis acid to polarise the carbonyl group and stabilise the oxyanion tetrahedral intermediate [74].

Given the relatively weak binding of the second zinc in the *B. cereus* (BcII) MBL and the fact that an important ligand holding the metal-ion to the protein is the bridging water which is consumed during the catalytic cycle of hydrolysis, it is possible that the second metal-ion could be lost during turnover. In fact, the kinetics of the hydrolysis of benzylpenicillin catalysed by the cobalt substituted β -lactamase from *B. cereus* (BcII) are biphasic with an initial burst of product formation followed by a steady-state rate of hydrolysis [75]. This was interpreted as being due to a branched kinetic pathway with two enzyme intermediate species, ES^1 and ES^2 , which have different metal: enzyme stoichiometries. ES^1 is a dicobalt enzyme intermediate and is catalytic, but is slowly losing one bound cobalt ion during turnover via the branching route, to give the mononuclear and inactive enzyme intermediate ES^2 . The dependence of enzyme activity on pH and metal-ion concentration indicates that only the dicobalt enzyme is catalytically active. The monocobalt enzyme species, formed during the catalytic cycle, is virtually inactive and requires the association of another cobalt ion for turnover. The dicobalt enzyme intermediate is responsible for the direct catalytic route, which is pH-independent between 5.5 and 9.5. The inactivation pathway of metal-ion dissociation occurs by both an acid catalysed and a pH-independent reaction, which is dependent on the presence of an enzyme residue of pK_a 8.9 in its protonated form and shows a large kinetic solvent isotope effect (H_2O/D_2O) of 5.2, indicative of a rate limiting proton transfer [75]. The pseudo first-order rate constant to regenerate the dicobalt β -lactamase from the monocobalt enzyme intermediate has a first-order dependence on cobalt ion concentration. This unusual behaviour is attributed to an intrinsic property of metallo hydrolytic enzymes that depend on a metal-bound water both as a ligand for the

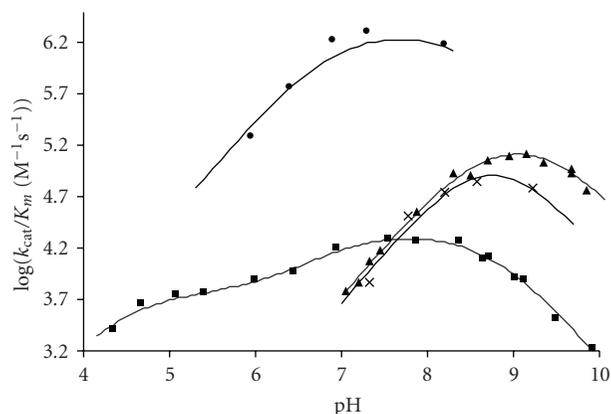


FIGURE 1: Plot of $\log k_{cat}/K_m$ for (■) ZnBCII-, (●) CoBCII-, (▲) CdBCII-, and (x) MnBCII-catalysed hydrolysis of cephalixin.

second metal-ion and as the nucleophile which is consumed during hydrolysis of the substrate and so has to be replaced to maintain the catalytic cycle [75, 76].

There remain questions regarding the necessity of the binuclear metal centre itself: (i) are metallo-enzyme species responsible for the physiological activity, the binuclear one or the mononuclear one, or are they interchangeable under certain conditions? and if so, (ii) are these changes relevant to any other functions, apart from catalysis, such as regulation of activity? It does seem intrinsically odd in the binuclear enzyme that the bridging hydroxide-ion is used as a nucleophile because of its presumed weak basicity. Hydroxide-ion has three lone pairs, two of which are taken up by metal-ion coordination and one of which is required for nucleophilic attack and covalent bond formation. If this bridging hydroxide-ion is used as the attacking nucleophile is it activated by general base catalysis so that concerted proton removal generates a dianionic tetrahedral intermediate?

REFERENCES

- [1] M. I. Page, "Structure-activity relationships: chemical," in *The Chemistry of β -Lactams*, M. I. Page, Ed., pp. 79–100, Blackie, London, UK, 1992.
- [2] M. I. Page, "The mechanisms of reactions of β -lactam antibiotics," *Advances in Physical Organic Chemistry*, vol. 23, pp. 165–270, 1987.
- [3] M. I. Page, P. Webster, and L. Ghosez, "The hydrolysis of azetidyl amidinium salts. Part 1. The unimportance of strain release in the four-membered ring," *Journal of the Chemical Society, Perkin Transactions 2*, no. 5, pp. 805–811, 1990.
- [4] A. M. Davis, P. Proctor, and M. I. Page, "Alcohol-catalysed hydrolysis of benzylpenicillin," *Journal of the Chemical Society, Perkin Transactions 2*, no. 8, pp. 1213–1217, 1991.
- [5] A. F. Martin, J. J. Morris, and M. I. Page, "Intramolecular general acid catalysis in the aminolysis of benzylpenicillin. A preferred direction of nucleophilic attack," *Journal of the Chemical Society, Chemical Communications*, no. 6, pp. 298–299, 1979.
- [6] N. P. Gensmantel and M. I. Page, "The aminolysis of penicillin derivatives. Rate constants for the formation and breakdown of the tetrahedral addition intermediate," *Journal of the*

- Chemical Society, Perkin Transactions 2*, no. 2, pp. 137–142, 1979.
- [7] J. J. Morris and M. I. Page, “Intra- and inter-molecular catalysis in the aminolysis of benzylpenicillin,” *Journal of the Chemical Society, Perkin Transactions 2*, no. 1, pp. 212–219, 1980.
- [8] A. Llinás, B. Vilanova, J. Frau, F. Muñoz, J. Donoso, and M. I. Page, “Chemical reactivity of penicillins and cephalosporins. Intramolecular involvement of the acyl-amido side chain,” *Journal of Organic Chemistry*, vol. 63, no. 24, pp. 9052–9060, 1998.
- [9] M. I. Page, “The energetics of intramolecular reactions and enzyme catalysis,” *Philosophical Transactions of the Royal Society B*, vol. 332, no. 1263, pp. 149–156, 1991.
- [10] N. P. Gensmantel, P. Proctor, and M. I. Page, “Metal-ion catalysed hydrolysis of some β -lactam antibiotics,” *Journal of the Chemical Society, Perkin Transactions 2*, no. 11, pp. 1725–1732, 1980.
- [11] S. Mölstad, C. S. Lundborg, A.-K. Karlsson, and O. Cars, “Antibiotic prescription rates vary markedly between 13 European countries,” *Scandinavian Journal of Infectious Diseases*, vol. 34, no. 5, pp. 366–371, 2002.
- [12] K. Poole, “Resistance to β -lactam antibiotics,” *Cellular and Molecular Life Sciences*, vol. 61, no. 17, pp. 2200–2223, 2004.
- [13] J. F. Fisher, S. O. Meroueh, and S. Mobashery, “Bacterial resistance to β -lactam antibiotics: compelling opportunism, compelling opportunity,” *Chemical Reviews*, vol. 105, no. 2, pp. 395–424, 2005.
- [14] G. A. Jacoby and L. S. Munoz-Price, “The new β -lactamases,” *The New England Journal of Medicine*, vol. 352, no. 4, pp. 380–391, 2005.
- [15] S. G. Waley, “ β -lactamase: mechanism of action,” in *The Chemistry of β -Lactams*, M. I. Page, Ed., pp. 198–228, Blackie, London, UK, 1992.
- [16] J.-M. Frère, “ β -lactamases and bacterial resistance to antibiotics,” *Molecular Microbiology*, vol. 16, no. 3, pp. 385–395, 1995.
- [17] D. J. Payne, “Metallo- β -lactamases—a new therapeutic challenge,” *Journal of Medical Microbiology*, vol. 39, no. 2, pp. 93–99, 1993.
- [18] M. Galleni, J. Lamotte-Brasseur, G. M. Rossolini, J. Spencer, O. Dideberg, and J.-M. Frère, “Standard numbering scheme for class B β -lactamases,” *Antimicrobial Agents and Chemotherapy*, vol. 45, no. 3, pp. 660–663, 2001.
- [19] B. A. Rasmussen and K. Bush, “Carbapenem-hydrolyzing β -lactamases,” *Antimicrobial Agents and Chemotherapy*, vol. 41, no. 2, pp. 223–232, 1997.
- [20] A. Felici, G. Amicosante, A. Oratore, et al., “An overview of the kinetic parameters of class B β -lactamases,” *Biochemical Journal*, vol. 291, part 1, pp. 151–155, 1993.
- [21] T. R. Walsh, S. Gamblin, D. C. Emery, A. P. MacGowan, and P. M. Bennett, “Enzyme kinetics and biochemical analysis of ImiS, the metallo- β -lactamase from *Aeromonas sobria* 163a,” *Journal of Antimicrobial Chemotherapy*, vol. 37, pp. 423–431, 1996.
- [22] A. Felici and G. Amicosante, “Kinetic analysis of extension of substrate specificity with *Xanthomonas maltophilia*, *Aeromonas hydrophila*, and *Bacillus cereus* metallo- β -lactamases,” *Antimicrobial Agents and Chemotherapy*, vol. 39, no. 1, pp. 192–199, 1995.
- [23] P. S. Mercuri, F. Bouillenne, L. Boschi, et al., “Biochemical characterization of the FEZ-1 metallo- β -lactamase of *Legionella gormanii* ATCC 33297^T produced in *Escherichia coli*,” *Antimicrobial Agents and Chemotherapy*, vol. 45, no. 4, pp. 1254–1262, 2001.
- [24] A. M. Simm, C. S. Higgins, A. L. Carenbauer, et al., “Characterization of monomeric L1 metallo- β -lactamase and the role of the N-terminal extension in negative cooperativity and antibiotic hydrolysis,” *Journal of Biological Chemistry*, vol. 277, no. 27, pp. 24744–24752, 2002.
- [25] A. Carfi, S. Pares, E. Duée, et al., “The 3-D structure of a zinc metallo- β -lactamase from *Bacillus cereus* reveals a new type of protein fold,” *The EMBO Journal*, vol. 14, no. 20, pp. 4914–4921, 1995.
- [26] O. Herzberg and P. M. Fitzgerald, in *Handbook of Metalloproteins*, A. Messerschmidt, W. Bode, and M. Cygler, Eds., vol. 3, pp. 217–234, John Wiley & Sons, Chichester, UK, 2004.
- [27] P. M. Fitzgerald, J. K. Wu, and J. H. Toney, “Unanticipated inhibition of the metallo- β -lactamase from *Bacteroides fragilis* by 4-morpholineethanesulfonic acid (MES): a crystallographic study at 1.85 Å resolution,” *Biochemistry*, vol. 37, no. 19, pp. 6791–6800, 1998.
- [28] T. A. Murphy, L. E. Catto, S. E. Halford, et al., “Crystal structure of *Pseudomonas aeruginosa* SPM-1 provides insights into variable zinc affinity of metallo- β -lactamases,” *Journal of Molecular Biology*, vol. 357, no. 3, pp. 890–903, 2006.
- [29] A. Carfi, E. Duée, M. Galleni, J.-M. Frère, and O. Dideberg, “1.85 Å resolution structure of the Zinc^{II}- β -lactamase from *Bacillus cereus*,” *Acta Crystallographica Section D*, vol. 54, part 3, pp. 313–323, 1998.
- [30] S. M. Fabiane, M. K. Sohi, T. Wan, et al., “Crystal structure of the zinc-dependent β -lactamase from *Bacillus cereus* at 1.9 Å resolution: binuclear active site with features of a mononuclear enzyme,” *Biochemistry*, vol. 37, no. 36, pp. 12404–12411, 1998.
- [31] N. O. Concha, C. A. Janson, P. Rowling, et al., “Crystal structure of the IMP-1 metallo β -lactamase from *Pseudomonas aeruginosa* and its complex with a mercaptocarboxylate inhibitor: binding determinants of a potent, broad-spectrum inhibitor,” *Biochemistry*, vol. 39, no. 15, pp. 4288–4298, 2000.
- [32] N. O. Concha, B. A. Rasmussen, K. Bush, and O. Herzberg, “Crystal structures of the cadmium- and mercury-substituted metallo- β -lactamase from *Bacteroides fragilis*,” *Protein Science*, vol. 6, no. 12, pp. 2671–2676, 1997.
- [33] R. Paul-Soto, R. Bauer, J.-M. Frère, et al., “Mono- and binuclear Zn²⁺- β -lactamase,” *Journal of Biological Chemistry*, vol. 274, no. 19, pp. 13242–13249, 1999.
- [34] D. de Seny, U. Heinz, S. Wommer, et al., “Metal ion binding and coordination geometry for wild type and mutants of metallo- β -lactamase from *Bacillus cereus* 569/H/9 (BcII),” *Journal of Biological Chemistry*, vol. 276, no. 48, pp. 45065–45078, 2001.
- [35] S. Wommer, S. Rival, U. Heinz, et al., “Substrate-activated zinc binding of metallo- β -lactamases,” *Journal of Biological Chemistry*, vol. 277, no. 27, pp. 24142–24147, 2002.
- [36] M. W. Crowder, Z. Wang, S. L. Franklin, E. P. Zovinka, and S. J. Benkovic, “Characterization of the metal-binding sites of the β -lactamase from *Bacteroides fragilis*,” *Biochemistry*, vol. 35, no. 37, pp. 12126–12132, 1996.
- [37] R. M. Rasia and A. J. Vila, “Exploring the role and the binding affinity of a second zinc equivalent in *B. cereus* metallo- β -lactamase,” *Biochemistry*, vol. 41, no. 6, pp. 1853–1860, 2002.
- [38] R. Paul-Soto, M. Hernandez-Valladares, M. Galleni, et al., “Mono- and binuclear Zn- β -lactamase from *Bacteroides fragilis*: catalytic and structural roles of the zinc ions,” *FEBS Letters*, vol. 438, no. 1–2, pp. 137–140, 1998.
- [39] W. Fast, Z. Wang, and S. J. Benkovic, “Familial mutations and zinc stoichiometry determine the rate-limiting step of

- nitrocefin hydrolysis by metallo- β -lactamase from *Bacteroides fragilis*," *Biochemistry*, vol. 40, no. 6, pp. 1640–1650, 2001.
- [40] M. Hernandez Valladares, A. Felici, G. Weber, et al., "Zn(II) dependence of the *Aeromonas hydrophila* AE036 metallo- β -lactamase activity and stability," *Biochemistry*, vol. 36, no. 38, pp. 11534–11541, 1997.
- [41] M. W. Crowder, T. R. Walsh, L. Banovic, M. Pettit, and J. Spencer, "Overexpression, purification, and characterization of the cloned metallo- β -lactamase L1 from *Stenotrophomonas maltophilia*," *Antimicrobial Agents and Chemotherapy*, vol. 42, no. 4, pp. 921–926, 1998.
- [42] J. Weston, "Mode of action of bi- and trinuclear zinc hydrolases and their synthetic analogues," *Chemical Reviews*, vol. 105, no. 6, pp. 2151–2174, 2005.
- [43] G. Parkin, "Synthetic analogues relevant to the structure and function of zinc enzymes," *Chemical Reviews*, vol. 104, no. 2, pp. 699–768, 2004.
- [44] N. P. Gensmantel, P. Proctor, and M. I. Page, "Metal-ion catalysed hydrolysis of some β -lactam antibiotics," *Journal of the Chemical Society, Perkin Transactions 2*, no. 11, pp. 1725–1732, 1980.
- [45] L. L. Kiefer and C. A. Fierke, "Functional characterization of human carbonic anhydrase II variants with altered zinc binding sites," *Biochemistry*, vol. 33, no. 51, pp. 15233–15240, 1994.
- [46] C. Mollard, C. Moali, C. Papamicael, et al., "Thiomandelic acid, a broad spectrum inhibitor of zinc β -lactamases," *Journal of Biological Chemistry*, vol. 276, no. 48, pp. 45015–45023, 2001.
- [47] A. Badarau, A. Llinás, A. P. Laws, C. Damblon, and M. I. Page, "Inhibitors of metallo- β -lactamase generated from β -lactam antibiotics," *Biochemistry*, vol. 44, no. 24, pp. 8578–8589, 2005.
- [48] D. W. Christianson and W. N. Lipscomb, "Comparison of carboxypeptidase A and thermolysin: inhibition by phosphoramidates," *Journal of the American Chemical Society*, vol. 110, no. 16, pp. 5560–5565, 1988.
- [49] S. Bounaga, A. P. Laws, M. Galleni, and M. I. Page, "The mechanism of catalysis and the inhibition of the *Bacillus cereus* zinc-dependent β -lactamase," *Biochemical Journal*, vol. 331, part 3, pp. 703–711, 1998.
- [50] N. O. Concha, B. A. Rasmussen, K. Bush, and O. Herzberg, "Crystal structure of the wide-spectrum binuclear zinc β -lactamase from *Bacteroides fragilis*," *Structure*, vol. 4, no. 7, pp. 823–836, 1996.
- [51] Z. Wang, W. Fast, and S. J. Benkovic, "Direct observation of an enzyme-bound intermediate in the catalytic cycle of the metallo- β -lactamase from *Bacteroides fragilis*," *Journal of the American Chemical Society*, vol. 120, no. 41, pp. 10788–10789, 1998.
- [52] Z. Wang, W. Fast, and S. J. Benkovic, "On the mechanism of the metallo- β -lactamase from *Bacteroides fragilis*," *Biochemistry*, vol. 38, no. 31, pp. 10013–10023, 1999.
- [53] S. McManus-Munoz and M. W. Crowder, "Kinetic mechanism of metallo- β -lactamase L1 from *Stenotrophomonas maltophilia*," *Biochemistry*, vol. 38, no. 5, pp. 1547–1553, 1999.
- [54] J. Spencer, A. R. Clarke, and T. R. Walsh, "Novel mechanism of hydrolysis of therapeutic β -lactams by *Stenotrophomonas maltophilia* L1 metallo- β -lactamase," *Journal of Biological Chemistry*, vol. 276, no. 36, pp. 33638–33644, 2001.
- [55] R. M. Rasia and A. J. Vila, "Mechanistic study of the hydrolysis of nitrocefin mediated by *B. cereus* metallo- β -lactamase," *ARKIVOC*, vol. 2003, no. 10, pp. 507–516, 2003.
- [56] R. Bicknell and S. G. Waley, "Cryoenzymology of *Bacillus cereus* β -lactamase II," *Biochemistry*, vol. 24, no. 24, pp. 6876–6887, 1985.
- [57] G. Garau, C. Bebrone, C. Anne, M. Galleni, J.-M. Frère, and O. Dideberg, "A metallo- β -lactamase enzyme in action: crystal structures of the monozinc carbapenemase CphA and its complex with biapenem," *Journal of Molecular Biology*, vol. 345, no. 4, pp. 785–795, 2005.
- [58] D. de Seny, C. Prosperi-Meys, C. Bebrone, et al., "Mutational analysis of the two zinc-binding sites of the *Bacillus cereus* 569/H/9 metallo- β -lactamase," *Biochemical Journal*, vol. 363, pp. 687–696, 2002.
- [59] Y. Yang, D. Keeney, X. Tang, N. Canfield, and B. A. Rasmussen, "Kinetic properties and metal content of the metallo- β -lactamase CcrA harboring selective amino acid substitutions," *Journal of Biological Chemistry*, vol. 274, no. 22, pp. 15706–15711, 1999.
- [60] S. Haruta, H. Yamaguchi, E. T. Yamamoto, et al., "Functional analysis of the active site of a metallo- β -lactamase proliferating in Japan," *Antimicrobial Agents and Chemotherapy*, vol. 44, no. 9, pp. 2304–2309, 2000.
- [61] J. D. Garrity, A. L. Carenbauer, L. R. Herron, and M. W. Crowder, "Metal binding Asp-120 in metallo- β -lactamase L1 from *Stenotrophomonas maltophilia* plays a crucial role in catalysis," *Journal of Biological Chemistry*, vol. 279, no. 2, pp. 920–927, 2004.
- [62] J. H. Ullah, T. R. Walsh, I. A. Taylor, et al., "The crystal structure of the L1 metallo- β -lactamase from *Stenotrophomonas maltophilia* at 1.7 Å resolution," *Journal of Molecular Biology*, vol. 284, no. 1, pp. 125–136, 1998.
- [63] M. Vanhove, M. Zakhem, B. Devreese, et al., "Role of Cys221 and Asn116 in the zinc-binding sites of the *Aeromonas hydrophila* metallo- β -lactamase," *Cellular and Molecular Life Sciences*, vol. 60, no. 11, pp. 2501–2509, 2003.
- [64] L. Hemmingsen, C. Damblon, J. Antony, et al., "Dynamics of mononuclear cadmium β -lactamase revealed by the combination of NMR and PAC spectroscopy," *Journal of the American Chemical Society*, vol. 123, no. 42, pp. 10329–10335, 2001.
- [65] C. Damblon, M. Jensen, A. Ababou, et al., "The inhibitor thiomandelic acid binds to both metal ions in metallo- β -lactamase and induces positive cooperativity in metal binding," *Journal of Biological Chemistry*, vol. 278, no. 31, pp. 29240–29251, 2003.
- [66] Z. Wang and S. J. Benkovic, "Purification, characterization, and kinetic studies of a soluble *Bacteroides fragilis* metallo- β -lactamase that provides multiple antibiotic resistance," *Journal of Biological Chemistry*, vol. 273, no. 35, pp. 22402–22408, 1998.
- [67] E. G. Orellano, J. E. Girardini, J. A. Cricco, E. A. Ceccarelli, and A. J. Vila, "Spectroscopic characterization of a binuclear metal site in *Bacillus cereus* β -lactamase II," *Biochemistry*, vol. 37, no. 28, pp. 10173–10180, 1998.
- [68] J. L. Myers and R. W. Shaw, "Production, purification and spectral properties of metal-dependent β -lactamases of *Bacillus cereus*," *Biochimica et Biophysica Acta*, vol. 995, pp. 264–272, 1989.
- [69] L. Bertini and C. Luchinat, "Cobalt(II) as a probe of the structure and function of carbonic anhydrase," *Accounts of Chemical Research*, vol. 16, no. 8, pp. 272–279, 1983.
- [70] L. I. Llarrull, M. F. Tioni, J. Kowalski, B. Bennett, and A. J. Vila, "Evidence for a dinuclear active site in the metallo- β -lactamase BcII with substoichiometric Co(II)," *Journal of Biological Chemistry*, vol. 282, no. 42, pp. 30586–30595, 2007.

- [71] P. A. Crawford, K.-W. Yang, N. Sharma, B. Bennett, and M. W. Crowder, "Spectroscopic studies on cobalt(II)-substituted metallo- β -lactamase ImiS from *Aeromonas veronii* bv. *sobria*," *Biochemistry*, vol. 44, no. 13, pp. 5168–5176, 2005.
- [72] P. A. Crawford, N. Sharma, S. Chandrasekar, et al., "Over-expression, purification, and characterization of metallo- β -lactamase ImiS from *Aeromonas veronii* bv. *sobria*," *Protein Expression and Purification*, vol. 36, no. 2, pp. 272–279, 2004.
- [73] J. D. Garrity, B. Bennet, and M. W. Crowder, "Direct evidence that the reaction intermediate of metallo- β -lactamase L1 is metal bound," *Biochemistry*, vol. 44, no. 3, pp. 1078–1087, 2005.
- [74] A. Badarau and M. I. Page, "The variation of catalytic efficiency of *Bacillus cereus* metallo- β -lactamase with different active site metal ions," *Biochemistry*, vol. 45, no. 35, pp. 10654–10666, 2006.
- [75] A. Badarau and M. I. Page, "Enzyme deactivation due to metal-ion dissociation during turnover of the cobalt- β -lactamase catalyzed hydrolysis of β -lactams," *Biochemistry*, vol. 45, no. 36, pp. 11012–11020, 2006.
- [76] A. Badarau, C. Damblon, and M. I. Page, "The activity of the dinuclear cobalt- β -lactamase from *Bacillus cereus* in catalysing the hydrolysis of β -lactams," *Biochemical Journal*, vol. 401, part 1, pp. 197–203, 2007.