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Metallo-β-lactamases: does it take two to tango?

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Contents

Abstract

Metallo-β-lactamases are a subset of zinc hydrolases able to hydrolyze the β-lactam ring of several antibiotics. The number of structural and mechanistic studies on these metalloenzymes has grown steadily in the recent years, due to their biomedical relevance in bacterial

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resistance. Crystallographic and spectroscopic studies on enzymes from different sources indicate that a conserved metal ligand set is able to bind either one of two metal equivalents. Even if both the mono- and bimetallic forms of these enzymes seem to be active in most cases, the binuclear species appear to be the most fit for conferring antibiotic resistance. The active nucleophile is a water/hydroxide molecule which has been found binding to either one or two Zn(II)s. The affinity for binding the second metal ion equivalent is related to the needs of the different enzymes in their natural environments. The exact role of the second Zn(II) is still unknown, but it has been proposed that it may help in positioning the substrate for the nucleophilic attack.

Keywords: Metallo-β-lactamases; Zinc; Antibiotics

1. Introduction

β-Lactams stand as the most important antibiotic family, and they represent one of the most useful chemotherapeutic weapons [1]. Antibiotic-based chemotherapy has proved to be successful by interfering with the enzymes responsible for cell wall peptidoglycan biosynthesis. This treatment does not produce secondary effects in humans due to the absence of peptidoglycan precursors in eukaryotic cells [2].

Antibiotic efficacy is continuously challenged by the emergence of resistant bacterial strains [3–6]. The fight against bacterial pathogens began early in the 1940s with the discovery of penicillin, the first β-lactam antibiotic. As early as 1946, a 6% penicillinase activity was detected in *Staphylococcus aureus* strains, which later (1950) amounted to 50%, and nowadays has raised to ca. 90% [1]. Since then, a continuous struggle between scientific efforts and bacterial resistance has been maintained. The incorporation of key antibiotics such as cephalosporins (during the 1960s) and carbapenems (in the present decade) has led to the development of further bacterial resistance mechanisms. One of the main weapons of bacterial resistance is the production of β-lactamases [3–5,7], enzymes capable of inactivating β-lactam antibiotics by cleaving their four-membered ring (Fig. 1). The more sophisticated antibiotic drugs are developed, the more resistant bacteria become. β-lactamase production is therefore associated with the appearance of a large number of resistant pathogenic bacterial strains.

Four classes of β-lactamases have been described and characterized. Three of them (A, C and D) comprise prototypical β-lactamases, which are serine-active enzymes, having distinct hydrolytic mechanisms as well as different substrate specificities [3–5,7].

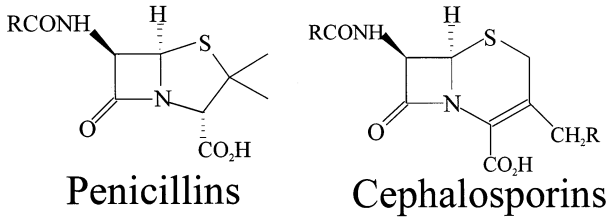
The latest chapter in this resistance saga has been written by a new class of enzymes, i.e. the metallo-β-lactamases, also called class B β-lactamases [5,8]. The native metal is Zn(II) in all cases, but these enzymes are also active when Co(II) or Cd(II) are introduced in their active site. These enzymes are characterized by their broad hydrolytic profile, i.e. they are able to hydrolyze penicillins, cephalosporins and carbapenems.

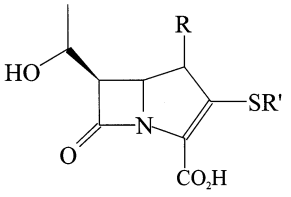
Metallo-β-lactamases represent a 7% of β-lactamases' universe. This apparently irrelevant proportion, however, cannot be overstated since they stand as the major

group of carbapenem-hydrolyzing enzymes [8]. Carbapenems, launched shortly after 3rd-generation cephalosporins, have the broadest spectra of all beta-lactams. Chromosomal metallo-β-lactamases with potent carbapenemase activity occur in *Stenotrophomonas maltophilia*, *Aeromonas hydrophila*, *Bacteroides fragilis* and *Flavobacterium odoratum* isolates. Plasmidic zinc carbapenemases were reported from *Pseudomonas aeruginosa*, *Serratia marcescens* and *Shigella flexneri* isolates [8–10]. Most of these strains are associated with post-surgery hospital infections.

In principle, co-administration of β-lactamase inhibitors to resistant bacteria is expected to restore the antibacterial activity of carbapenems. However, the existing clinically useful inhibitors are ineffective against metallo-β-lactamases, indicating the high potential risk of their existence.

Structural and biochemical studies on serine-active β-lactamases have been of relevance for the understanding of their enzymatic action [3,4]. New efforts are focused in tracing a structure-function relationship in the metallo-β-lactamase family. A recent review has focused on the microbiological and biochemical aspects of these enzymes [8]. Here we will review the recent structural studies of this newly characterized class of metalloenzymes from a bioinorganic perspective.





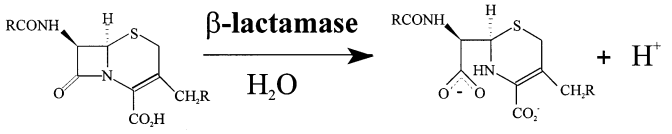


Fig. 1. Structure of the basic skeleton of prototypical β-lactam antibiotics, and schematic reaction hydrolysis of a β-lactam ring.

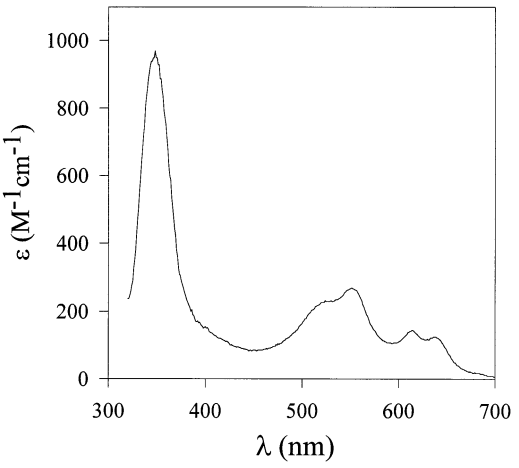


Fig. 2. Electronic spectrum of Co(II),Co(II)–βLII at pH 6.0 in 20 mM sodium succinate + 1 M NaCl. The enzyme concentration was 0.38 mM. The spectrum of the apoenzyme was substracted.

2. Crystallographic and spectroscopic studies: counting the zincs

The first report of a metallo β-lactamase dates back to 1966, in which the existence of two β-lactamases from the soil bacterium *Bacillus cereus* (569/H/9) was reported [11]. They were generically termed β-lactamase I and II (βLI and βLII hereafter) [11–15]. βLII was shown to be inhibited by EDTA, thus indicating the essential role of a metal ion, identified as Zn(II) [13,14]. The enzymatic activity could be partially recovered upon addition of Co(II), Ni(II) or Cd(II), whereas the presence of Cu(II) or Hg(II) did not render an active enzyme [13]. βLII was also peculiar for being able to hydrolyze cephalosporins. Later, a mutant strain from *Bacillus cereus* (5/B/6) was shown to produce a βLII-like enzyme, but no serine-active β-lactamase [15]. However, βLII was considered as a biochemical curiosity for almost two decades, until homologous metallo-β-lactamases were detected in harmful bacteria [8].

Metal substitution was fundamental in obtaining a first picture of the metal site structure, as for many other zinc enzymes [16–19]. Early NMR experiments in the apo, Zn(II) and Co(II)-substituted βLII were useful for defining the involvement of three His residues in the metal binding site [20–22]. Substitution of the native Zn(II) by Co(II) gave rise to a characteristic Cys → Co(II) LMCT at 348 nm (Fig. 2) [13]. Less intense features were observed in the visible region at 530, 551, 614 and 637 nm, attributable to ligand field transitions [23–25], with intensities being typical of tetrahedral high spin Co(II) complexes. All these data seemed to indicate the existence of a tetrahedral metal site embedded in a His_3Cys coordination polyhedron. However, this picture was not compatible with the existence of a catalytic zinc site, which should possess at least one coordinated water molecule (i.e. the attacking nucleophile, as shown in Fig. 1). For this reason, Bicknell et al. suggested

the possibility of a pentacoordinated Co(II) site based in MCD and EPR evidence, with three His, a Cys and a solvent molecule as ligands [25].

This reasoning may be questioned by the fact that the visible region of the spectrum of Co(II)–βLII is remarkably similar to that of Co(II)-carbonic anhydrase at alkaline pH [17,18], which possesses a His_3ZnOH active site [26]. It is then interesting to compare these spectra with the ones from a His_3Cys tetrahedral site engineered in human carbonic anhydrase II [27,28]. When Thr 199, normally hydrogen bonded to the metal-bound water molecule in carbonic anhydrase, is mutated onto a Cys, this residue coordinates apically the metal ion [28]. The electronic spectrum of Co(II)–T199C carbonic anhydrase exhibits the typical LMCT band at 350 nm, and d–d transitions in the visible range which, even being typical of a tetrahedral Co(II) site, differ from the ones of the native site [27]. By these means, it may be concluded that the spectral similarity between Co(II)-carbonic anhydrase and Co(II)–βLII cannot be fortuitous, but only due to highly similar coordination environments.

On the other hand, early spectroscopic measurements and equilibrium dialysis experiments had witnessed the existence of two metal binding sites for Cd(II) and Co(II) in βLII [14,22,23]. Later, a low-resolution structure of Cd(II)-substituted βLII indicated the probable binding of more than one ion equivalent in the metal site [29]. However, the existence and the role of the second binding site was dismissed for some time.

By the end of 1995, the crystal structure of βLII at pH 5.7 was reported at a 2.5-Å-resolution [30]. This structure revealed an active site containing one Zn(II) coordinated to three histidine residues (His 86, His 88 and His 149) and to a solvent molecule in a tetrahedral fashion (Fig. 3), bearing a strong resemblance to the carbonic anhydrase active site [26]. These data were clearly against the spectroscopic evidences in the Co(II)-substituted enzyme [14,23,35]: no Cys ligands were found, and the enzyme contained a mononuclear site. These discrepancies could be attributed in principle to differences in the coordination spheres of the Zn(II)- and Co(II) enzymes, and this structure seemed to fill the gap in the discussion about the metal site coordination mode.

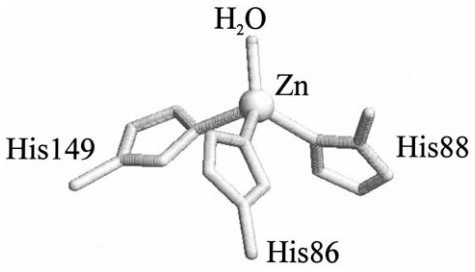


Fig. 3. Schematic view of the metal site of βLII, from Carfi et al. (file 1bmc, Protein Data Bank, Brookhaven) [30]. The picture was rendered with RasMol v2.6 (R. Sayle (1994), Greenford, Middlesex, UK).

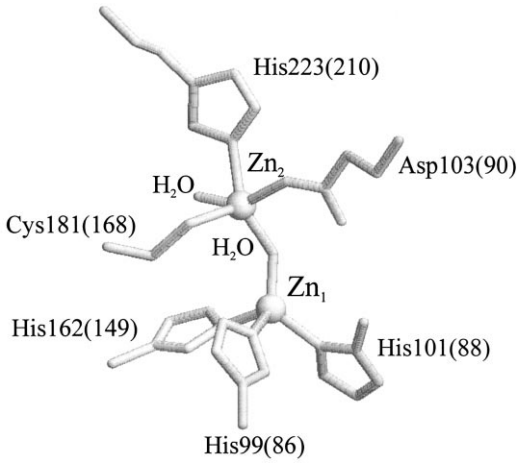


Fig. 4. Schematic view of the metal sites of *Bacteroides fragilis* β-lactamase, from Concha et al. [31] (file 1znb, Protein Data Bank, Brookhaven). The picture was rendered with RASMOL v2.6 (R. Sayle (1994), Greenford, Middlesex, UK). The amino acid numbering given in parenthesis for the picture of the *B. fragilis* enzyme indicate the corresponding numbering in βLII.

This paper was the kick-off for the current interest in structural studies on metallo-β-lactamases, stimulated by the finding of metallo-β-lactamases in pathogenic bacteria. Since then, several papers reporting crystal structures of metallo-β-lactamases have appeared [30–39], and fifteen high resolution structures have been deposited in the Protein Data Bank, eleven of which are available to date. This fact should not be surprising by itself, but it is puzzling to realize that most of these structures only concern two different enzymes, i.e. βLII and the β-lactamase from *Bacteroides fragilis* (*Bf* βL hereafter).

The subsequent elucidation of the 3D structure of the metal-dependent β-lactamase from *Bacteroides fragilis* (*Bf* βL) provided even more controversy [31]. This enzyme possesses an active site with two Zn(II) ions (Fig. 4): one of them (Zn_1) exhibits a coordination polyhedron superimposable with the only Zn(II) found in βLII (Fig. 3). The second one (Zn_2) displays a trigonal bipyramidal coordination geometry, with a solvent molecule as a μ-hydroxo or μ-aquo bridging ligand between the two ions (Fig. 4). Zn_2 is also coordinated to an Asp, a Cys, a His, and an additional solvent molecule. Surprisingly, all the Zn_2 protein ligands found in *Bf* βL are conserved in βLII (Scheme 1) [40]. In this way, a similar second binding site could be also expected in the latter enzyme. Moreover, these data clearly proved that a Cys residue was acting as a metal ligand. Later, a crystal structure on Cd(II)-substituted *Bf* βL also revealed a bimetallic site almost similar to the Zn(II) native one, giving a strong support to the validity of metal substitution spectroscopic analyses [33].

Several research groups were then prompted to find and characterize the elusive second binding site in βLII. Different estimations of the metal binding affinities of apo-βLII indicated a smaller affinity for divalent cations of the second metal site

[22,23,41]. The metal binding constants seem to be highly dependent on the pH and buffer conditions. There is now a general agreement that the absence of the second metal ion equivalent in βLII could be due to the looser or negligible metal binding at this site at the crystallization conditions.

A recent study has selectively probed each one of the two metal sites in βLII by metal substitution and different spectroscopic methods [41]. Addition of Co(II) to the mono-Zn(II) βLII enzyme yields the hybrid Zn(II),Co(II)–βLII species, without displacing the Zn(II) ion from the first site. In this way, the Co(II),Co(II)- and Zn(II),Co(II)-substituted enzymes can be separately studied. The spectrum shown in Fig. 2 displays the spectral features of the two metal sites filled with Co(II). The intense d–d bands (with ε values larger than 200 M^{-1} cm^{-1}) indicate a carbonic anhydrase-like tetrahedral site, i.e. Zn_1. On the other hand, the 348 nm band is due to the Cys residue coordinating the second metal ion, as can be seen in the spectra of the hybrid Zn(II),Co(II)–βLII (Fig. 5). The low-intensity ligand field band ($\lambda = 520$ nm, $\varepsilon \approx 20$ M^{-1} cm^{-1}) observed for the latter species (obscured by the intense absorption of the tetrahedral site in the Co(II),Co(II)-derivative) indicate an octahedral geometry for this site. The recently reported spectrum of Co(II),Co(II)-substituted *Bf* βL closely resembles that of Co(II),Co(II)–βLII [42].

Paramagnetic NMR spectra of Co(II),Co(II)- and Zn(II),Co(II)–βLII further confirmed the presence of a Cys ligand in the second site, as well as the existence of four bound His residues [41] (Fig. 6). Three of them are coordinating the first (tetrahedral) site, whereas the fourth one is a ligand of the second site. A number

	76	96
B.cereus	QKRVTDVII	T**H**A**H**A**D**RIGGIK
B.fragilis	HAKVTTFIP	N**H**W**H**G**D**CIGGLG
S.marcescens	YKIKGSIS	S**H**F**H**S**D**STGGIE
A.hydrophila	RKPVLEVIN	T**N**Y**H**T**D**RAGGNA
S.maltophilia	TPRDLRLILLS	**H**A**H**A**D**HAGPVA

	147	170
B.cereus	KG**H**TEDNIVVWLPQYN	ILVGG**C**L
B.fragilis	GG**H**ATDNIVVWLPTEN	ILFGG**C**M
S.marcescens	PG**H**TPDNVVVWLPERK	ILFGG**C**F
A.hydrophila	PA**H**TPDGIFVYFPDEQ	VLYGN**C**I
S.maltophilia	AG**H**TPGSTAWTWTDTRNGKPVRIAYAD**S**L	

	202	215
B.cereus	NINAVVPG**H**GE	VGD
B.fragilis	SARYVVPG**H**GD	YGG
S.marcescens	KAKLVVPS**H**SE	VGD
A.hydrophila	KIKTVIGG**H**DSPLHG	
S.maltophilia	PCDVLLTP**H**PGASNW	

Scheme 1.

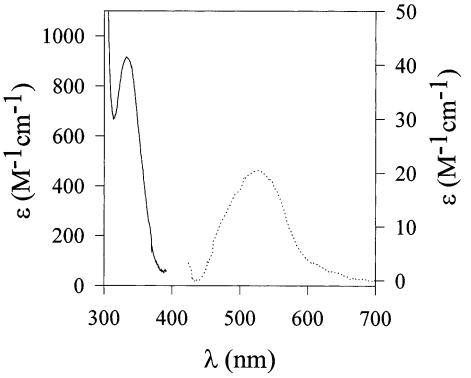


Fig. 5. Electronic spectrum of Zn(II),Co(II)–βLII obtained by addition of Co(II) to the mono-Zn(II) βLII adduct at pH 6.0 in 20 mM sodium succinate + 1 M NaCl. The solid trace (at the left) corresponds to a sample of 0.15 mM enzyme reconstituted with 20 mM Co(II). The spectrum of mono-Zn-βLII was substracted in the 300–400 nm region. The dotted trace (at the right) is the spectrum of 8 mM enzyme with 9 mM added Co(II).

of resonances are absent in the Zn(II),Co(II)–βLII spectrum, and they can be readily attributed to the metal ligands of the high affinity site. These observations concur with the ligand environment observed in the crystal structure of the *B. fragilis* enzyme [31] (Fig. 4). By these means, it was shown that, under proper conditions, a binuclear site can be built also in βLII and *Bf* βL [41].

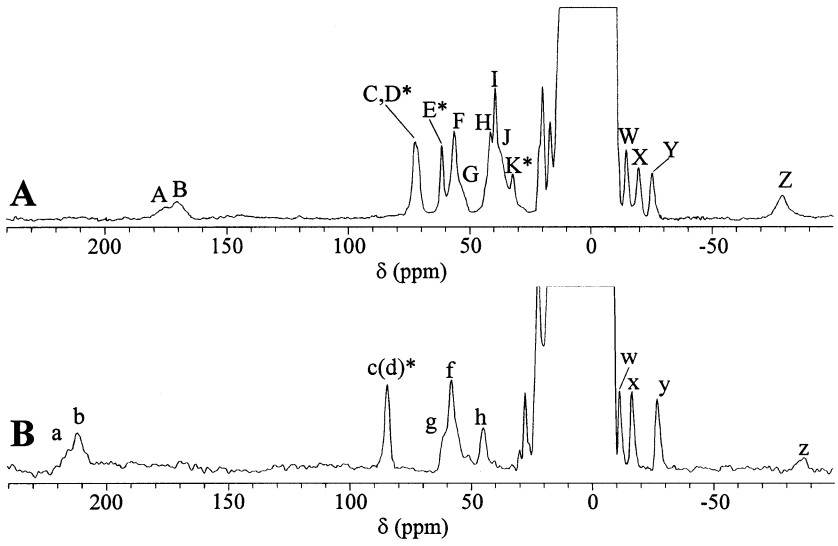


Fig. 6. ^{1}H-NMR spectra of (A) Co(II),Co(II)–βLII, and (B) Zn(II),Co(II)–βLII. Signals marked with an asterisk are absent when the spectra are recorded in D_2O, being assigned as His NHs. Resonances A,B (a,b) correspond to the β-CH_2 of Cys 168. The spectra were recorded in H_2O at 200 MHz and 298 K in 20 mM sodium succinate + 1 M NaCl at pH 6.0.

The coup-de-grace in this telltale story was given by the very recent reports of two high resolution structures of βLII in which two metal ions are located in the active site, with the same coordinating residues as found in the *Bf* βL enzyme [34,35]. In both cases, βLII crystals were grown at higher Zn(II) concentrations (100–500 μM). Notwithstanding, this second zinc ion is more loosely bound (see below).

The structure of the tetrameric β-lactamase from *Stenotrophomonas maltophilia* (referred to as L1) showed a bimetallic active site, but with a different metal ligand set for Zn_2 [38]. This metal ion is bound to an Asp, two His and two water ligands, one of them acting as bridging ligand with Zn_1 (see below).

The β-lactamase from *Aeromonas hydrophila* AE306 deserves a separate chapter [43,44]. This enzyme is able to bind two metal ions, but with strikingly different affinities [44]. The mono-Zn(II) enzyme is active (and it exhibits exclusively carbapenemase activity). When the second equivalent is bound, it acts as a noncompetitive inhibitor of the enzymatic activity. Again, the metal affinities are strongly dependent on the pH. Fluorescence and circular dicroism spectra indicate that the metal uptake is accompanied by conformational changes. In addition, the incorporation of Zn(II) stabilizes the enzyme against thermal and urea-induced denaturation [44]. Since His 86 is replaced by an Asn in the *A. hydrophila* enzyme (Scheme 1), the active site is expected to present distinctive features. More structural and spectroscopic data are strongly awaited in this peculiar β-lactamase.

3. The crystal structures in detail: another turn of the screw

The available crystal structures revealing binuclear zinc sites exhibit some differences which are analyzed below. Table 1 summarizes some selected data from these structures.

3.1. The Bacteroides fragilis enzyme

The structures reported by Concha et al. at pH 7.0 for the Zn(II) and the Cd(II)-substituted enzymes display similar metal sites [31,33]. When Hg(II) is added to the Zn(II) enzyme, Zn_2 is no longer bound, and the Hg(II) ion binds between the former liganded Cys 181 and Cys 104 [33]. This observation explains the fact that mercury compounds are able to inactivate the enzyme.

The two Zn(II) ions are located 3.5 Å apart in the native enzyme [31], with the bridging hydroxide at a bonding distance both from Zn_1 and Zn_2. However, in the structure from Carfi et al. (at pH 9.0), the zinc ions are 3.4 Å apart, but without any bridging ligand (Fig. 7) [32]. A water molecule is found beyond the coordination sphere of Zn_1. This metal ion then adopts a curious tricoordinated geometry (Fig. 7). This is a rather remarkable result, since the position of the water molecule seems to be the only striking difference between the metal site structures at different pH values (Fig. 7).

Table 1
Selected structural parameters from the available X-ray structures of metallo-β-lactamases

Enzyme	Ref.[a]	Resolution (Å)	pH	r_{Zn-Zn} (Å)	r_{Zn_1-W} (Å)[b]	r_{Zn_2-W} (Å)[b]
βLII	[30] (1bmc)	2.5	5.7	–	2.31	–
	[34] (1bme)	1.85 (100 K)[c]	5.6	3.74	2.25	–
	[35] (1bc2)	1.9 (100 K)[c]	5.2	3.85	1.90	2.48
				4.36	1.90	3.06
Bf βL	[31] (1znb)	1.85	7.0	3.46	1.97	2.13
				3.47	2.88	2.06
	[32] (2bmi)	2.0	9.0	3.35	3.42	
				3.38	3.54	
Cd(II)–*Bf* βL	[33] (2znb)	2.15	7.0	3.71	2.10	2.75
				3.71	1.97	2.15
Hg(II)–*Bf* βL	[33] (3znb)	2.55	7.0	4.78[d]	4.20	–
Bf βL–BPT	[37] (1a8t)	2.55	6.6	2.84	–	–
Bf βL–MES	[36] (1a7t)	1.85	7.5	3.5	1.85	2.21
				3.48	1.93	2.15
L1	[38]	1.7 (100 K)[c]	7.75	3.4	1.86	2.07

[a] The protein data bank accession code is indicated in parenthesis.
[b] W stands for the bridging water/hydroxide.
[c] Temperature for data collection.
[d] Zn–Hg distance.

Two crystal structures of the *Bf* βL enzyme with inhibitors have been reported during the last year. One of them shows how a MES molecule coming from the crystallization buffer is positioned establishing hydrogen bonds with the zinc site [36]. MES, however, is only a weak inhibitor. On the other hand, a strong inhibitory effect was observed by biphenyl tetrazoles. A 2.5 Å structure of one of these inhibitors complexed with the enzyme indicates that the bimetallic structure is preserved [37], with the only exception that there is no neat electron density at the position of the bridging water molecule, which is replaced by the tetrazole moiety. These results strongly indicate that the bridging hydroxide plays a key role in the catalytic mechanism.

3.2. βLII

The structure from Carfi et al. was solved at 1.85 Å in cryocooled crystals at 100 K [34]. The two Zn(II) ions are 3.7 Å apart, and there is no bridging water molecule. A Zn_1-bound water is found in a similar orientation as the non-bound solvent molecule in the *Bf* βL structure reported by the same group [32](cf. Fig. 7). This situation entails Zn_1 a bipyramidal trigonal geometry with a missing equatorial ligand. A carbonate ion coordinates Zn_2 in a bidentated fashion, and one of the carbonate oxygens is hydrogen bonded to Wat1. Both Zn_2 and the carbonate ion have been refined with a 0.6 occupancy and B factors of 22 $Å^2$.

Fabiane et al. reported a βLII structure at pH 5.2 solved at 1.9 Å and 100 K [35]. The Zn_1–Zn_2 distances are longer than in the *Bf* enzyme, and the position of the second Zn(II) differs in the two molecules found in the asymmetric unit, without any noticeable effect on the Zn_1 coordination. This effect is attributed to the loose binding of this metal ion. In contrast with the previously discussed data, the Zn_1 environment is clearly tetrahedral in both molecules. A water molecule is strongly bound to Zn_1 and it is hydrogen bonded to Asp 90, like in the *Bf* enzyme. Based on these results, the authors rule out the involvement of Zn_2 in water activation.

One of the main differences in the active site surroundings between βLII and the *Bf* enzyme is that Arg 91 is replaced by Cys 87. The guanidinium group in βLII is located at 4.2 Å from Zn_2, and it has been repeatedly suggested that its presence could impair the metal binding in the second site [31,32,34,35]. This hypothesis still remains to be tested.

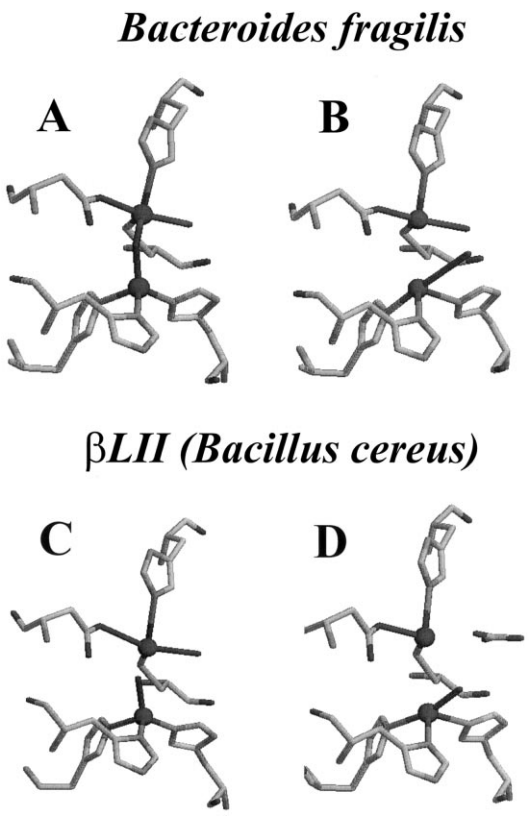


Fig. 7. Comparative schematic views of the metal sites of (A) *Bf* β-lactamase, taken from Concha et al. [31] (file 1znb); (B) *Bf* β-lactamase, taken from Carfi et al. [32] (file 1bmi); (C) βLII, taken from Fabiane et al. [35] (file 1bc2); (D) βLII, taken from Carfi et al. [34] (file 1bme). All data files are from the Protein Data Bank, Brookhaven. The pictures were rendered with RasMol v2.6 (R. Sayle (1994), Greenford, Middlesex, UK).

3.3. Apo-βLII

The apo-enzyme presents the same overall architecture as the holoenzyme [34]. The metal ligands in the 86–90 loop are the most affected, yielding a more solvent-exposed active site. According to the authors, the Zn(II) may also play a structural role in holding the active site and its surroundings.

3.4. The L1 enzyme

The structure of the L1 metallo-β-lactamase from *Stenotrophomonas maltophilia* revealed a binuclear zinc site [38]. The Zn_1 coordination geometry is superimposable to the one of βLII and *Bf* βL. This is not the case for Zn_2, since a Ser residue replaces the more ubiquitous Cys ligand (Scheme 1). This residue does not bind the metal ion, but it is hydrogen bonded to the second coordinating water. On the other hand, a new His ligand is found, which replaces the non-coordinating Arg 91(in βLII) or Cys 87 (in *Bf* βL), thus arising from a different protein region (Scheme 1). These changes impose a distorted pentacoordinated geometry to Zn_2, as will be discussed in Section 4.

4. A coordination chemistry look into the active site

The active sites of metallo-β-lactamases possess a unique coordination environment as compared with other binuclear zinc enzymes. First, a Cys ligand is present in βLII and *Bf* βL, which is itself an unusual feature in catalytic zinc sites in proteins [45–48]. In addition, these are the only binuclear zinc centers with a coordinated Cys residue.

The geometry of Zn_1 is tetrahedral. Its His_3OH coordination environment resembles the one of carbonic anhydrase [26] and fits into a general zinc binding motif, with three histidine residues in a His-X-His sequence, and a third, distant His ligand (cf. Scheme 1) [45,46]. However, a subtle difference should be pointed out. The two His residues close in the primary sequence (94 and 96 in human carbonic anhydrase) are normally bound through their Nε2 atom [26], in order to relief side chain sterical hindrance between the proximal histidine ligands. However, in βLII, the coordinating nitrogens for the proximal histidines are Nε2 for His 86, and Nδ1 for His 88. This situation is quite curious, since $N\delta 1$ coordination seems to be favored only for His residues distant in the primary sequence [46]. A thorough analysis regarding these preferences is available for mononuclear zinc sites [46,48], but this trend is also valid for binuclear centers [47].

Zinc is well known for being a borderline Lewis acid in terms of the HSAB theory, being able to coordinate N, O, and S- donor ligands [17]. The polyhedron coordination of Zn_2 in βLII and *Bf* βL is unique in possessing all the four types of ligands being present in zinc enzymes, namely: a His, a Cys, an acidic residue (Asp) and a water molecule. Moreover, oxygen, sulfur and nitrogen-donor atoms are simultaneously coordinating Zn_2 in this family of zinc enzymes. The L1 enzyme is

an exception [38]. In this case, Zn_2 displays a $His_2Asp(H_2O)_2$ trigonal bipyramidal coordination geometry due to the Cys→Ser and Arg→His replacements. Moreover, the plane defined by the equatorial ligands (Wat 1, His 89 and His 225) is rotated by 76° around the Zn_2–Wat 1 axis with respect to the one in *Bf* βL (defined by Wat 1, His 223, and Cys 181). This alteration is clearly due to the point mutations in a similar fold over the active site. The functional needs for this different coordination environment are not known.

The Zn_1–Zn_2 distances are rather long (cf. Table 1), as compared with other binuclear zinc enzymes such as those from the aminopeptidase family [47,48]. This may be due to the existence of only one bridging ligand (when found) joining the two metal ions, the μ-hydroxo moiety. The absence of a bridging protein ligand is another uncommon feature, since binuclear zinc sites most often possess carboxylate bridging moieties (Asp, Glu) in this position.

5. Mono versus binuclear functionality: how many zincs are needed?

At the current status of knowledge of β-lactamase structure, one may wonder whether the binding of two metal ions is relevant for catalysis or not. We will now briefly consider some mechanistic studies.

Apo-βLII is able to recover the activity of the holoenzyme by re-incorporating Zn(II) [14]. The stepwise addition of Zn(II) (and also Co(II)) to apo-βLII clearly reveals two inflection points when the β-lactamase activity is measured vs. the added metal ion concentration [14,41]. The binding of the first metal equivalent yields an active enzyme, but the following uptake of a second metal ion equivalent renders an enzyme with its maximum activity. This is valid both for Zn(II) and Co(II).

In the case of *Bf* βL, the situation is different. Both sites do also show a higher affinity towards metal ions [42]. The full activity is restored upon incorporation of two metal equivalents to the apoenzyme, but it is not possible to monitor the sequential occupancy of the two sites [42]. In contrast, a mono-Zn(II) enzyme has been obtained by extensive dialysis against buffer solutions, which lead to the selective loss of Zn_2 [49]. Surprisingly, the mononuclear enzyme displayed a hydrolytic activity which compares to that of the binuclear one. In addition, a hybrid Zn(II),Cd(II) adduct proved to be more efficient than the bi-Zn(II) *Bf* enzyme against cefoxitin [49].

The Cys181Ser mutation in *Bf* βL yielded an impaired enzyme, in which only Zn_1 is bound [39]. The X-ray structure of this mutant reveals that the hydrolytic water/hydroxide is farther away from Zn_1 than in the wild type enzyme [39]. The results from these two recent papers [39,49], when put together, indicate that the Cys residue is fundamental for binding Zn_2, but that indeed plays a key role in the catalytic action of the mononuclear derivative. Hence, in the binuclear L1 enzyme, where the Cys is naturally replaced by a Ser, an additional ligand (His 89) is needed to construct the second metal site [38].

The pH-dependence of the mono-Zn(II) βLII enzymatic activity has been studied [50,51]. The k_{cat}/K_m values were shown to depend on three ionizable groups: two of them with overlapping pK_a values around 5.6, and the third one with a pK_a of 9.5 [51]. The acidic deprotonations have been tentatively assigned to the zinc-bound water and Asp 90. Typically, water molecules coordinate Zn(II) and by this means they display pK_a values considerably lower than bulk water, even at the expense of decreasing the nucleophilicity of the bound water/hydroxide. This apparent drawback is compensated by an increase the effective hydroxide concentration at neutral pH. The observed pK_a value is, however, lower than in other zinc enzymes [17]. In the *Bf* βL enzyme, no pH dependence was observed for k_{cat} and k_{cat}/K_m between 5.25 and 10.0 [42]. This may indicate that the water pKa could be lower than 5.25, a fact that could be explained by assuming that the nucleophile is bound to the two Zn(II) ions.

Asp 90 is postulated to act as a general base after the nucleophilic attack performed by the zinc-bound hydroxide [51]. This residue 90 has been shown to be essential, since mutation of this residue by Asn or Glu yields non-active enzymes [52]. According to this proposal, a dianionic intermediate is formed, which presumably involves a pentacoordinated Zn(II) (bound to the substrate during the nucleophilic attack). Recent kinetic [42] and spectroscopic [53] evidences support the existence of an intermediate during the hydrolysis of nitrocefin, purportedly, a negatively charged metal-bound species.

Evidence for the metal-substrate interaction are available from a series of elegant, low temperature kinetic experiments performed by Bicknell and coworkers more than a decade ago [24,25]. These studies may now be appraised at the light of the available structural data. Three enzyme-substrate intermediates of the benzylpenicillin hydrolysis by Co(II)–βLII were characterized by electronic and MCD spectroscopy [25]. Their spectra differed from the spectrum of the resting state Co(II) enzyme, indicating that the metal coordination sphere of Co_1(which gives rise to the most intense d–d bands) is altered during the catalysis. Substrate coordination to Co_1 is then likely to occur. A substantial intensity enhancement of the Cys-Co(II) LMCT band during hydrolysis was also observed even at -50°C, indicating that the Co_2 environment participates actively in catalysis.

The separate study of the mono and binuclear species in βLII may not always be achievable. The formation of a tetrahedral adduct with ligand field features different from the ones of the resting state enzyme was detected during the titration of apo-βLII vs. Co(II) at pH 6.0 [41]. This intermediate later rearranges upon further Co(II) addition to yield the resting state conformation. Fluorescence-monitored Co(II) incorporation also reveals a conformational change at pH 6.0 which is not present at pH 7.0 (unpublished data from our laboratory). These observations indicate that even when the second metal site is vacant, its ligand set may be exploited by Co_1. This hypothesis deserves to be further explored. In any case, flexibility is an issue in β-lactamase, and in this case, it clearly involves both metal ions. The different positioning of Zn_2 in the crystal structure of βLII is significant at this respect [35].

We should also consider that the different crystal structures have cast some doubt on the existence of a bridging hydroxide in βLII [34,35], and even in the *Bf* enzyme [32] (Fig. 7). EPR experiments on the Co(II)-substituted *Bf* enzyme have shown that the two bound Co(II) ions are high spin and uncoupled, giving support to this possibility [42]. It is quite clear that the mono- and binuclear species must obey different catalytic mechanisms. The existence of an active binuclear metal site without a bridging water/hydroxide raises the possibility of a third catalytic mechanism.

Lipscomb and Sträter have recently reviewed the different structural characteristics of mononuclear and binuclear zinc peptidases [47], which may be relevant to the β-lactamase case. A carboxylate ion is usually found hydrogen bonded to the catalytic water in mono-zinc enzymes. The presence of this residue is essential for the enzyme activity, acting as a general base and proton shuttle as well as in stabilizing the transition state. In the mono-Zn(II) βLII, Asp 90 seems to be the best candidate for performing this role [51].

Binuclear zinc peptidases, instead, possess a water molecule bridging the two metal ions, and one empty coordination site at each of the two Zn(II). Both Zn(II) may participate in nucleophile activation, as in leucine aminopeptidase [54], and this situation may be analogous to the *Bf* enzyme, assuming the presence of a strong bridging hydroxide. Substrates do bind to one or both the metal ions during catalysis in peptidases [47]. In the present case, the presence of Zn_2 may be fundamental in positioning the β-lactam ring for a proper nucleophilic attack [35].

6. Concluding remarks: the best is yet to come

β-Lactamases stand as one of the best examples of how bacteria have evolved their own defence mechanisms, and clearly metallo-β-lactamases belong to the latest generation of bacterial resistance. A plethora of recent, exciting data in the literature have addressed the metallo-β-lactamase issue by using different, sometimes complementary approaches. Most of the initial questions may be unanswered yet, e.g. the postulation of a detailed mechanism, but significant progress has been achieved.

Now it is well established that all metallo-β-lactamases may bind two metal ions, and it still remains to elucidate how the protein environment fine tunes the binding affinities and the role of the different ligands. The different behaviors displayed by these enzymes (the *A. hydrophila* β-lactamase may constitute a third case) follow the distinct binding efficiencies of Zn_2. The *Bf* enzyme appears then to be evolutionary more fit for β-lactamase hydrolysis, indicating that the building of the second site is by itself advantageous for the enzyme. Pathogenic bacteria are nowadays under a stronger selective pressure than *B. cereus* for displaying antibiotic resistance, giving support to this appealing hypothesis. Mechanistic, spectroscopic and crystallographic studies on these enzymes in their native and mutated forms are expected to provide a deeper insight into these enzymes' function in the coming years.

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