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Review

Metallo-β-lactamases: two binding sites for one catalytic metal ion?

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Abstract. During the past few years the results from molecular biological, biochemical, chemical, physical and theoretical approaches expanded the knowledge about metallo-β-lactamases considerably. The main reason for the attracted interest is a persisting medical problem. Bacteria expressing metallo-β-lactamases can be resistant to treatment with all the known β-lactam antibiotics, and they are additionally invulnerable to combined treatment with inhibitors for the wider-spread serine-β-lactamases. However, clinically useful inhibitors for metallo-β-lactamases are not yet available. In spite of the rapidly expanding knowledge base a central question is still controversially discussed: is it the mononuclear, the binuclear or the metal-free state which might serve as the physiologically relevant target for inhibitor design? A summary of the present views of the roles and coordination geometries of metal ion(s) in metallo-β-lactamases is combined with a discussion of the possibly variable metal ion content under physiological conditions.

Key words. Metallo-β-lactamase; zinc; antibiotic resistance; inhibition; EXAFS.

Dedication

This article is dedicated to the memory of Rogert Bauer. He established perturbed angular correlation of gamma rays spectroscopy as a valuable tool in the study of metallo proteins. It was Rogert who first raised the title question a few years ago. He was a great source of support and inspiration for both of us. We will remember him as an outstanding personality both as a scientist and as a friend.

Introduction

β-lactam compounds inhibit transpeptidases in bacterial cell wall biosynthesis, rendering them one of the most successful classes of antibiotics. Bacteria, however, have evolved different escape strategies against β-lactam compounds, including the production of β-lactamases. These enzymes inactive the antibiotics by hydrolyzing the C-N bond of the β-lactam ring. Four classes (A–D) of the enzyme family are known. Only class B, the metallo-β-lactamases require zinc ions for catalytic activity.

The first metallo-β-lactamase identified was found in a non-pathogenic strain of *Bacillus cereus* [1]. For a long time metallo-β-lactamases were considered interesting but clinically unimportant. This view changed when the first metallo-β-lactamase-producing pathogenic bacteria were found. Their number is continuously increasing, and some of them are considered to be of high clinical importance [2].

During the past 5 years several reviews highlighted the emergence and epidemiology of metallo-β-lactamases

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[2–7], therapeutic [8] and evolutionary [7] aspects as well as inhibition [6], structure and mechanisms [9, 10]. After a historical synopsis, recent work on metallo-β-lactamases with an emphasis on the structure and function of the catalytic zinc binding site will be discussed, with special attention to work published since 1999.

A major motivation for intensified research efforts is the urgent need to keep β-lactam antibiotics, especially carbapenems, as useful clinical therapeutics. This requires strategies to deactivate metallo-β-lactamases. The availability of three-dimensional (3-D) structures of target proteins is most welcome as a basis for the application of computational tools as well as for quantum mechanical approaches to inhibitor design. The imperfect definition of physicochemical properties of metal ions in general still causes problems in both ab initio computational approaches and docking experiments performed with metallo enzymes. The availability of crystal structures of metallo-β-lactamases seemed to promise a precise target definition at a very early stage of the research. The comparison of different structures allowed the identification of functionally crucial amino acid side chains and led to the classification of class B β-lactamases into subclasses B1–B3. One common feature of the subclasses is the conserved structural motif of two zinc binding sites in close proximity, apparently forming a binuclear zinc site. Based on the first two crystal structures published, the seemingly straightforward classification into mononuclear zinc-β-lactamases such as BcII from *B. cereus* [11] and binuclear enzymes such as CcrA from *Bacteroides fragilis* [12] had to be revised after it became obvious that both enzymes were active in the mono- and binuclear states [13, 14]. This finding immediately provoked the question where the metal ion of the mononuclear species is located during catalysis, an issue which is of outstanding importance e. g. in the choice of starting models for any computational approach. For historical reasons and because similar carboxypeptidases were better understood, the 3-His zinc binding site was usually considered to be the primary catalytic site. Therefore, docking and quantum mechanical studies as well as model system studies either used the binuclear enzymes or located the catalytic zinc ion of mononuclear model systems in the 3-His site. Combinations of different biophysical techniques, however, showed that a static view of the mononuclear enzymes is an oversimplification, and meanwhile even the primary catalytic site assignment appears questionable.

Historical development of the field

The first metallo-β-lactamase from *Bacillus cereus* strain 569/H/9 (BcII) was reported in 1966 [1]. Involvement of a thiolate group in zinc binding was discovered shortly after [15], followed by a systematic investigation of metal cofactor requirements. This resulted in the view that the metal ion is needed for activity and that at least two metal ions can bind to the protein, with a lower affinity for a second metal ion. A cysteine residue of the protein was identified as a metal ion ligand [16]. By ^{1}H-nuclear magnetic resonance (NMR) spectroscopy it was possible to identify four histidine residues which are involved in metal ion binding, assigning three histidines to a so-called tighter binding site and a fourth histidine to a second zinc binding site [17, 18]. The appearance of sulphur-to-cobalt charge transfer bands in spectroscopic studies on Co(II)-substituted enzyme led to the suggestion that a thiol ligand might be involved in metal binding at the first site [19]. In 1985 the amino acid sequence of the *B. cereus* enzyme was published [20], and a second representative of the growing enzyme family was purified from *Pseudomonas maltophilia* and shown to be a tetramer (L1) [21]. The use of cryo-enzymology identified changes in the coordination geometry of the active site metal ion in Co(II)-substituted BcII during catalysis [22, 23]. Results from an initial X-ray crystallographic investigation were presented in 1987 [24], but it took another 8 years before a high-resolution structure was published [11]. In 1989 a study with metal-substituted BcII and a thiono-β-lactam substrate provided evidence for a possibly direct interaction of substrate and metal ion during catalysis [25]. In 1991 two new members of the family, both obtained from human pathogens, were identified, namely the enzymes from *Bacteroides fragilis* (CcrA) [26] and *Aeromonas hydrophila* (CphA) [27]. In 1994 an enterobacterial enzyme was identified in a clinical isolate of *Serratia marcescens* (Imp-1) [28]. At that time a method for rapid identification of metallo-β-lactamases was published [29]. One year later the plasmid-mediated dissemination of the metallo-β-lactamase gene blaIMP (Imp-1) among clinically isolated strains of *Serratia marcescens* was demonstrated in Japan [30].

In 1995 the first detailed 3-D structure of BcII from *Bacillus cereus* was published [11]. The structure was obtained from crystals grown at pH 5.6 and showed that a single zinc ion was bound by three histidines, thus confirming the earlier predictions [17, 18]. In 1996 carbapenem-resistant strains of *Pseudomonas aeruginosa* were found to carry the blaIMP gene [31, 32]. In the same year a crystal structure of CcrA was published [12]. In contrast to the BcII structure [11] a binuclear zinc site with a bridging hydroxide ion was identified, which gave rise to the suggestion of an alternative catalytic mechanism making use of both zinc ions. At the same time a spectroscopic investigation led to the conclusion that CcrA required two zinc ions for catalysis [33]. In 1997 a series of mercaptoacetic acid thiol ester derivatives were shown to be inhibitors of metallo-β-lactamases but proved to have vastly deviating efficiencies against dif-

ferent representatives of the enzyme family [34, 35]. Low molecular weight thiol compounds were shown to inhibit IMP-1 efficiently [36]. The detailed characterization of CphA from *A. hydrophila* demonstrated that full activity was reached with only one bound zinc ion and furthermore that a second bound zinc ion non-competitively inhibited this enzyme [37]. In 1998 the field started to explode. New members of the enzyme family were found in *Chryseobacterium meningosepticum* (BlaB) [38] and *Shigella flexneri* [39], a mechanism of catalysis for BcII was suggested [40], a crystal structure of L1 [41], additional structures of BcII [42, 43] and CcrA [44] were published, as well as first structures of CcrA with bound inhibitors [45, 46]. The metal ion requirement of CcrA was controversially discussed [13, 47], and structural comparisons resulted in the finding that the zinc-binding motif of metallo-β-lactamases is additionally found in glyoxalase II and arylsulphatases [48].

Subclasses and superfamily

To date 27 coordinate files of metallo-β-lactamases have been deposited in the Protein Data Base covering 8 different enzymes comprising examples for all the three subclasses, partly in complex with different inhibitors. The PDB accession codes, the respective organism, the crystallographic resolution, the corresponding reference and short comments concerning the structures are summarized in table 1.

The structural data together with sequence data for additional enzymes resulted in the suggestion to subdivide class B into the three subclasses B1–B3. To facilitate the comparison of enzymes from different origins, a standard numbering scheme was suggested [49]. A recent alignment of all the known crystal structures provides an update of the standard numbering scheme, now localizing structurally conserved parts of the amino acid sequences [50]. According to the latter suggestions the enzymes were classified according to the set of metal ion ligands in the two binding sites as follows:

- Subclass B1 is characterized by zinc binding site 1, composed of three His residues and zinc binding site 2, composed of one His, one Cys and one Asp.
- Subclass B2 is characterized by zinc binding site 1, with one of the histidines replaced by an asparagine residue. Zinc binding site 2 is unmodified compared to subclass B1. Assignments of putative zinc ligands for subclass B2 enzymes resulted from sequence alignments and have been challenged by extended X-ray absorption fine structure (EXAFS)-spectroscopic and site-directed mutagenesis studies [51, 52] (see be-

Table 1. Crystal structures of metallo-β-lactamases solved to date.

Subclass	PDB code	Enzyme	Resolution [Å]	Ref.	Comments
B1	1BMC	BcII	2.50	[11]	first structure published, 1 zinc ion bound by 3 His residues
B1	1BVT	BcII	1.85	[43]	binuclear zinc site, bound bicarbonate ion
B1	1BC2	BcII	1.90	[42]	
B1	2BC2	BcII	1.70	–	
B1	3BC2	BcII	1.70	–	mononuclear 3 His, 1 water site with oxidized cysteine
B1	1DXK	BcII	1.85	[59]	structure of a Cys168Ser mutant
B1	1MQO[a]	BcII	1.35	–	Cd-substituted enzyme
B1	1ZNB	CcrA	1.85	[12]	first structure of a binuclear enzyme, Zn-bridging hydroxide
B1	2ZNB	CcrA	2.15	[144]	cadmium-substituted enzyme
B1	3ZNB	CcrA	2.70	[144]	mercury-substituted enzyme
B1	4ZNB	CcrA	2.65	[66]	Cys181Ser mutant
B1	2BMI	CcrA	2.00	[44]	
B1	1A7T	CcrA	1.85	[45]	complex with MES
B1	1A8T	CcrA	2.55	[46]	complex with a biphenyl tetrazole
B1	1HLK	CcrA	2.50	–	complex with a tricyclic inhibitor bound
B1	1KR3	CcrA	2.50	[115]	complex with a tricyclic inhibitor bound
B3	1SML	L1	1.70	[41]	first structure of a tetrameric enzyme
B1	1DD6	IMP-1	2.00	[118]	complex with mercaptocarboxylate
B1	1DDK	IMP-1	3.10	[118]	native enzyme
B1	1JJE	IMP-1	1.80	[128]	succinic acid-inhibited enzyme
B1	1JJT	IMP-1	1.80	[128]	succinic acid-inhibited enzyme
B3	1JT1	Fez-1	1.78	[134]	D-captopril-inhibited enzyme
B3	1KO7	Fez-1	1.65	[134]	native enzyme
B1	1M2X	BlaB	1.50	[133]	D-captopril-inhibited enzyme
B1	1KO2	Vim-2	2.20	–	cysteine oxidized
B1	1KO3	Vim-2	1.91	–	cysteine reduced
B2	1PX1[a]	CphA	1.70	–	first structure of subclass B2

[a] Coordinates on hold until publication.

low). The recently obtained crystal structure of CphA from *A. hydrophila* (1PX1) shows the single zinc ion exclusively bound in the Cys-Asp-His site of the enzyme [O. Dideberg and G. Garau, personal communication].

– Subclass B3 has the same ligands in zinc binding site 1 as subclass B1, but the cysteine ligand of subclasses B1 and B2 in binding site 2 is replaced by a histidine.

This classification was recently challenged. New sequence alignments resulted in the suggestion to group subclasses B1 and B2 into one new subclass and separate them from the present subclass B3 due to the absence of any detectable relationship of B3 with B1 + B2 at the sequence level [53]. The latter article additionally gives a summary of the presently available gene sequences.

A comparison of the available structures for representatives from different organisms is shown in figure 1. It becomes obvious that the global fold within the subclasses is very similar. For all four representatives of subclass B1 the positions of the zinc ions and their ligands show very little variation. The metallo-β-lactamase structure is composed of two parts of the molecule which are connected by a loop spanning residues 191–200. Part 1 contains the highly conserved sequence motif His116-X-His118-X-Asp120 of zinc ligands. This motif is also conserved within the metallo-β-lactamse superfamily. The global fold of representatives from subclass B3 shows minor differences compared to subclass B1, whereas the zinc ligand set is modified. Cys-221, which is conserved in subclasses B1 and B2, is replaced by a serine. The third zinc ligand in site 2, however, is now an additional histidine, namely His-121, and thus the immediate neighbor of Asp-120, which is also a zinc ligand. This variation has consequences for the structural organization of the protein and for the charge distribution at the metal ion binding site(s). The subclass B1 structure is organized such that the two parts of the molecule are connected via the

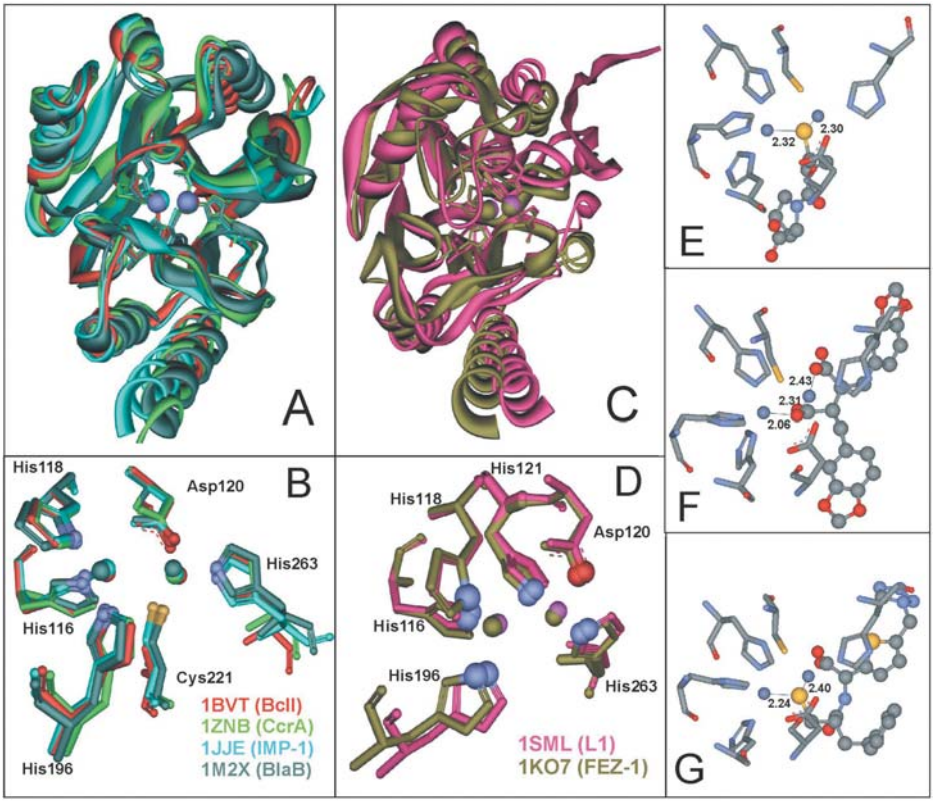

Figure 1. Structures of metallo-β-lactamases. Panels *A* and *B* show a structural alignment of four different representatives from subclass B1, namely the enzymes from *Bacillus cereus* (BcII, accession code 1BVT), *Bacteriodes fragilis* (CcrA, accession code 1ZNB), *Pseudomonas aeruginosa* (IMP-1, accession code 1JJE) and *Chryseobacterium meningosepticum* (BlaB, accession code 1M2X). Panels *C* and *D* represent the subclass B3 enzymes from *Stenotrophomonas maltophilia* (L1, accession code 1SML) and *Fluoribacter gormanii* (FEZ-1, accession code 1KO7). The lower panels (*B* and *D*) represent the zinc ions (spheres) and the ligating side chains (sticks) with the directly coordinating atoms as spheres. The structural alignment was performed by choosing the C_α positions of the zinc ligands His116, His118 and Asp120 as tethers. Panels *E*–*F* represent the binding mode of inhibitors exemplified for BlaB (1M2X) with bound D-captopril in panel *G*, IMP-1 with a bound biaryl succinic acid (1JJT) in panel *F* and IMP-1 with a bound mercaptocarboxylate inhibitor (1DD6) in panel *E*. The zinc-coordinating side chains are represented as stick models, and the bound inhibitors are shown as ball-and-stick models. The distances of inhibitor atoms binding to zinc are given in angstroms.

zinc ions, with part 1 contributing two histidine ligands for Zn1 and one aspartate for Zn2. The third histidine for Zn1 is contributed by part 2 of the molecule, which also contributes the cysteine and the histidine ligand for Zn2. In subclass B3 structures both zinc ions are coordinated by two residues from part 1 each, and one ligand for each of the zinc ions is contributed by part 2. The effect of the ligand set modification is that the negatively charged sulphur ligand to Zn2 of subclass B1 is now replaced by a neutral histidine. A close-up look at the zinc ion binding sites is shown in figure 1B and D.

In various studies site-directed mutagenesis was used to investigate the role of individual residues in catalysis. As representatives of subclass B1 various mutants of BcII from *B. cereus* [14, 54–62], CcrA from *Bacteroides fragilis* [33, 63–66] and IMP-1 from *P. aeruginosa* [58, 67–69] were studied. As representatives of subclass B2, putative metal ion ligands of CphA, the enzyme from *A. hydrophila*, were mutated [52]. For the subclass B3 enzyme L1 from *Stenotrophomonas maltophilia* various mutants were tested [70–72], and Asp-120 was shown to play an important role in proton transfer reactions [71]. Besides the metal ion ligands, a flexible loop frequently harboring a solvent-exposed tryptophan residue and comprising residues 60–66 in the consensus numbering scheme was shown to be involved in substrate recognition and binding [58, 73–76]. Besides the artificially produced mutants numerous naturally occuring variants of known enzymes have been described. For example, for the plasmid-borne enzyme of the IMP-1 type, the following variants obtained from clinical isolates have been described during the last 4 years: IMP-2 [77], IMP-3 [78], IMP-4 [79], IMP-5 [80], IMP-6 [81], IMP-7 [82], IMP-8 [83], IMP-10 [84], IMP-11, IMP-12 [85] and IMP-13 [86].

Using sequence comparisons, characteristic motifs of metallo-β-lactamases could be detected which were also found in other proteins, thus giving rise to the metallo-β-lactamase superfamily [48, 87]. The family was soon extended to include class B β-lactamase, glyoxalase II, arylsulphatase, flavoprotein, cyclase/dehydrase, a messenger RNA (mRNA) 3′-processing protein, a DNA cross-link repair enzyme, a DNA uptake-related protein, an alkylphosphonate uptake-related protein, CMP-N-acetylneuraminate hydroxylase, the romA gene product, alkylsulphatase and insecticide hydrolases [88]. Recently it was shown that ElaC encodes a binuclear zinc phosphodiesterase [89, 90]. The ubiquitously expressed nuclear protein Artemis belongs to the metallo-β-lactamase superfamily and defines a new group, β-CASP, within this family by acting on nucleic acids [91–93]. The common sequence motifs together with the conserved zinc binding site are found in proteins with vastly deviating functions. In several cases the function of the metallo-β-lactamse-like domain is still unclear.

If, however, active site structures of zinc enzymes are compared instead of amino acid sequences or protein folds, the metallo-β-lactamase active site appears far less 'peculiar' than initially expected. Other enzyme families, such as the amino peptidases, show binuclear zinc sites as well [94]. This makes the metallo-β-lactamase family even more interesting. The metallo-β-lactamases might be considered model zinc enzymes due to the fact that dynamic aspects of the zinc-protein interaction entered the focus of research interests. It is no longer the activated water molecule alone which is considered to be the dynamic entity but likely the metal ion itself which undergoes rapid ligand exchange reactions which might be considered to be of functional importance for enzyme activity.

Biophysical and theoretical approaches

Results from different biophysical techniques proved to complement crystallographic results in the development of ideas about the enzyme mechanisms, namely NMR, perturbed angular correlation (PAC) of γ-rays and EXAFS spectroscopy.

The early availability of the crystal structure of a metallo-β-lactamase from *B. cereus* with only one [11] zinc ion bound advanced the suggestion of a mononuclear hydrolysis mechanism. Shortly after, the crystal structure of the *Bacteroides fragilis* enzyme showed a binuclear zinc site with a bridging hydroxide ion and led to the suggestion of an alternative binuclear mechanism [12]. It became even more puzzling when a new crystal structure of BcII, obtained from crystals grown at a higher pH, also showed a binuclear zinc site [43]. A first systematic investigation of the metal ion dependence of the catalytic activity for BcII resulted in the surprising finding that this enzyme was active as the mono- and the binuclear enzyme and obviously used two different catalytic mechanisms in the two possible states [14]. A similar result was obtained for the enzyme from *B. fragilis* [13], but the corresponding results contradiced to a later study with the same enzyme which concluded in cooperative binding of zinc ions and suggested that the di-zinc form was the only relevant form for catalysis [65]. For the mono-zinc enzyme the 3-His site was obviously considered to be the 'catalytic site' of BcII [43].

PAC of γ-ray spectroscopy provides information on metal ion coordination geometry through measurement of the nuclear quadrupole interaction (NQI) between the nuclear electric quadrupole moment and the electric field gradient from the surrounding charge distribution. A structural investigation of Cd-substituted BcII by PAC spectroscopy resulted in the unexpected result that a single cadmium ion when bound to the binuclear binding site is distributed between both sites. Surprisingly, it was

not the 3-His site but the Cys-Asp-His site which was preferentially occupied (70%) [55]. The assignment of binding sites to the two different NQIs observed resulted from the investigation of a Cys168Ala mutant which showed only one NQI corresponding to the 3-His site of the enzyme. A Zn/Cd hybrid enzyme proved to exclusively bind zinc and cadmium ions in the 3-His and the Cys-Asp-His site, respectively.

To our knowledge these results were the first to demonstrate the distribution of a catalytic metal ion between two different binding sites. The functional role of a metal ion distribution between two binding sites during catalysis, however, remains to be resolved.

Based on the results obtained with the cadmium-substituted enzyme, studies with the native zinc enzyme were initiated. One of the few spectroscopic techniques to directly allow observation of the environment of zinc ions is EXAFS spectroscopy. The method results in structural information about the nature, number and distance of zinc binding atoms. Additional information about second sphere atoms in a distance of up to 4.5 Å in case of e.g. coordinating histidine residues as well as about the rigidity of the active site structure in terms of Debye-Waller factors can be obtained. Initial detailed investigations of mononuclear Zn-BcII resulted in a partially occupied Cys-Asp-His site [14], and a later improved investigation of mono- and bi-zinc BcII resulted in an almost 1:1 distribution of a single bound zinc ion between both available binding sites [54]. This result implies that the two binding sites are characterized by two almost identical microscopic dissociation constants. In the case of BcII, however, a second zinc ion was found to bind considerably weaker to the enzyme compared to a first equivalent bound.

The comparison of X-ray structures of BcII obtained under different conditions reveals an apparent variation in ligand-to-metal distances. A similar variability of ligand-to-metal distances was not observed in EXAFS spectra obtained for Zn_1- and Zn_2-BcII wild-type and various Zn_2-mutant enzymes [54]. It was suspected that the crystallographically studied BcII species might have been partially occupied with zinc ions. In the case of partly occupied sites, Zn_1 and Zn_2 species might coexist in the crystals [43]. Additionally, slightly differing ligand positions in occupied and unoccupied sites have been observed by comparison with a structure of the metal-free enzyme [43]. The distribution of zinc between the two sites in the Zn_1 enzyme, as has been demonstrated by EXAFS spectroscopy of Zn_1 enzymes [14, 54, 95], might result in average electron densities, leading to incorrect positions of residues lying between the 'real' positions in occupied and unoccupied sites [54]. In contrast to X-ray crystallography, unoccupied sites do not contribute to the data in EXAFS spectroscopy. Therefore only very small variations in ligand-to-metal distances are observed. This resulted in a very precise definition of the average distance of histidine nitrogen to zinc of 1.993 ± 0.019 Å, and a distance of the presumable hydroxide ion bound to zinc of 1.73 ± 0.03 Å is found in the 3-His site [54]. Only one X-ray structure (1BMI/A) shows such a short distance [44], which led to the suggestion to reconsider the choice of distance constraints for metal ion-bound hydroxides in crystallographic refinements [54].

During the last few years several groups initiated theoretical investigations to learn more about the electronic structure of the enzymes' active sites [96–104], substrate [75, 105–108], inhibitor binding [106, 109, 110] and about their catalytic mechanism [75, 105, 106, 111, 112]. Based on crystallographic data, starting models of varying complexity were generated for molecular dynamics, quantum mechanical calculations and docking studies. In general, two alternative starting models were used. For simulations of the mononuclear enzyme the zinc ion was placed in the 3-His site of BcII [104, 105, 107, 111]. Binuclear enzymes were simulated either starting from binuclear structures of the enzymes from *B. fragilis* (CcrA) [97, 98, 100, 108–110], *Bacillus cereus* (BcII) [98] or *Pseudomonas aeruginosa* (IMP-1) [106]. It was shown that the carbonyl group of the β-lactam ring might directly coordinate to zinc, e.g. [111] or substrate interaction with zinc takes place via an activated hydroxide ion, e.g. [75]. All the different starting models chosen ultimately resulted in plausible binding modes or reaction mechanisms. Depending on the models and methods used, different amino acid side chains appeared to be responsible for e.g. the protonation of the lactam nitrogen. Until now no theoretical approach considered that the metal ion of mononuclear species might change its binding site during a catalytic cycle, although theoretical approaches showed a very flat energy landscape for the exact location of the metal ions in a binuclear model [101, 106]. In a site-directed mutagenesis study of BcII, all the metal ligands of both zinc sites were selectively replaced by non-coordinating residues [54, 57]. All the mutants were still able to bind two zinc ions [54] and showed some activity as binuclear enzymes. Only a Cys221Ala was virtually inactive as a mononuclear enzyme but recovered activity when binding a second zinc ion [14, 57]. Since all the mutants show reduced activities compared to the wild type for both the mono- and binuclear states, it might be assumed that both sites are required for activity even if only one zinc ion is bound. Early time-resolved cryo-spectroscopic investigations of Co(II)-substituted BcII [22, 23], most likely performed with the Co_1 enzyme (compare [54]), resulted in a strong increase of the sulphur-to-cobalt charge transfer band intensity upon binding of benzylpenicillin [23]. A new interpretation of these data suggested that the Co(II) ion in the mononuclear enzyme might change its preferred binding site upon binding of substrate [54]. Since it has been clearly shown that

a single bound metal ion is distributed between both binding sites [51, 54, 55, 113] it appears arbitrary to consider the metal ion as being locked in one of the available sites for a computational approach. One study, however, suggested not assigning a primary catalytic site [75]. It could be demonstrated for the Cd(II)-substituted enzyme that a single bound metal ion changes its binding site without interaction with substrates on a microsecond time scale [113]. It might be worth testing whether a translocation during the catalytic cycle of the enzymes could be part of the catalytic mechanism. This is also important since data obtained recently for the subclass B2 enzyme from *A. hydrophila* (CphA) suggest that the metal ion of the mononuclear enzyme is preferentially bound in the Cys-Asp-His site of the protein [51, 52], which supports earlier findings demonstrating full occupation of the single metal ion with a Cys sulphur as a ligand [95, 114]. But even for CphA it became apparent that the single metal ion in the Cd(II)-substituted enzyme shows two alternative ligand geometries [51]. The functional role of metal ion translocation in mononuclear species thus remains to be resolved.

Inhibition of metallo-β-lactamases

A number of different inhibitors for metallo-β-lactamases were described. They belong to different substance classes, namely tricyclic natural products from *Chaetomium funicola* [115], trifluoromethyl alcohols and ketones [116], hydroxamic acids [117], mercaptocarboxylates [51, 118–120], biphenyl tetrazoles [46], carbapenem derivatives [121, 122], cephamycins and moxalactam [123], thiols [40, 119, 120, 124, 125], cysteinyl peptides [125], inhibitors derived from single-domain antibody fragments elicited in the *Camelidae* [126], thioesters [34, 119, 124] phenazines from a *Streptomyces* [127], 2,3-disubstituted succinic acid [128], sulphonylhydrazones [129], disulphides [130], tioxo-cephalosporin derivatives [131] and penamaldic derivatives of penicillins [131, 132]. In particular, the substituted succinic acids [128] and mercapto-carboxylic inhibitors [118, 119] are potent inhibitors with inhibition constants in the nanomolar range. Several structures with bound inhibitors have been solved. Figure 1E–G represents three examples for different inhibitor types and demonstrates generally found features for inhibitor binding. In most structures known to date, the bound inhibitors contribute a new ligand or new ligands for the zinc ions and result in the replacement of the zinc-bound water molecules. In most cases thiol-containing compounds show a direct interaction of thiolate sulphur with both metal ions of the binuclear enzymes. Until now, however, no clinically useful compound could be identified. Furthermore it turned out that the affinity of different metallo-β-lactamases for the same inhibitor molecule can deviate vastly. Only for one representative, namely D-captopril, are structural data for the inhibited complexes of all the three subclasses available. A crystal structure of BlaB, the subclass B1 enzyme from *Chryseobacterium meningosepticum*, showed the thiol group of D-captopril coordinating to both zinc ions as a bridging ligand (fig. 1E) [133]. A crystal structure of the subclass B3 enzyme FEZ-1 from *Fluoribacter gormanii*, however, only showed unspecific binding some distance from the zinc ions [134]. An investigation combining ultraviolet-visible (UV-vis) spectroscopy of Co(II)-substituted with PAC and EXAFS spectroscopy of Cd(II)-substituted, D-captopril-inhibited mononuclear species of the enzymes BcII (subclass B1) and CphA (subclass B2) from *Bacillus cereus* and *A. hydrophila*, respectively, resulted in subclass-specific differences. Both EXAFS and PAC spectroscopy with Cd_1-BcII resulted in distribution of the single metal ion between both binding sites. Binding of D-captopril to BcII did not lock the metal ion in one of the two binding sites but only shifted their relative occupation [51]. EXAFS spectroscopy showed the sulphur of D-captopril to coordinate to Cd(II) when located at the 3-His site of BcII, which was further supported by UV-vis spectroscopic results with the Co(II)-substituted enzyme [51]. For the subclass B2 enzyme CphA from *A. hydrophila* a PAC investigation for Cd_1-CphA clearly demonstrated distribution of the single cadmium ion bound between two structurally different binding sites with a strong preference for the Asp-Cys-His site. Binding of D-captopril, however, led to the identification of a single well-defined coordination geometry for cadmium which was clearly identified as the Asp-Cys-His site with D-captopril sulphur as the fourth zinc ligand by EXAFS spectroscopy [51]. For the dinuclear enzyme L1 from *Stenotrophomonas maltophilia* (subclass B3) the inhibitor binding mode has been investigated by combining results from EXAFS at the zinc-K edge and UV-Vis spectroscopy of the Co^{2+}-substituted L1 enzyme. The results suggested that the thiolate of D-captopril directly coordinates within the metal binding site of Zn_2-L1. A new charge transfer band appearing in the inhibited Co_2 enzyme also indicates coordination of the sulphur to the dimetal centre. The captopril sulphur appears to bind by replacing a previously metal-bound water molecule without drastic changes in the overall coordination geometry. But in contrast to the crystal structure of D-captopril-inhibited BlaB [133], the D-captopril sulphur does not bridge the two zinc ions but only coordinates to one of them [unpublished data]. Structural data for five different D-captopril-inhibited enzymes thus demonstrated five different binding modes of the same compound.

An additional interesting effect of inhibitor binding concerns the influence of bound ligands on metal ion affinities. The presence of D-captopril proved to modify the

affinity of BcII for Cd(II). The dissociation constant for a first cadmium ion bound dropped by a factor of 3, whereas a second cadmium ion bound by a factor of 10 weaker compared to the uninhibited enzyme [51]. A detailed investigation of the binding of thiomandelate to Cd-substituted BcII resulted in the opposite finding. A combined Cd^{113}-NMR and PAC spectroscopic study of the enzyme at various cadmium/enzyme stoichiometries resulted in data which suggested that it was only possible to obtain the binuclear inhibited enzyme and not a mononuclear inhibited species. The inevitable conclusion of the authors was that the presence of thiomandelate induced positive cooperativity of cadmium binding for BcII [135]. The latter findings reinforce the question how many zinc ions are required and what is the physiologically relevant enzyme species to be considered as a target in inhibitor design.

Mono- or binuclear zinc enzymes under physiological conditions?

Only a few studies exist concerning the role of bound zinc ions for the stability of metallo-β-lactamases. In a crystal structure of metal-free BcII some structural variation in the active site compared to the metal-loaded enzyme [43] could be observed. The ^{1}H-^{15}N heteronuclear single quantum coherence spectra of the backbone amides of BcII indicated clear structural differences between metal-free, mono- and binuclear enzymes [113]. For the enzyme from *A. hydrophila* CphA fluorescence emission and circular dichroism spectra revealed slight conformational changes upon titration of the apoenzyme with Zn^{2+} ions. And differential scanning calorimetry transitions and intrinsic fluorescence emission spectra in the presence of increasing concentrations of urea demonstrated that the catalytic zinc strongly stabilizes the conformation of the enzyme, with the di-Zn enzyme being even more resistant to thermal and urea denaturation than the mono-Zn enzyme [37]. For glyoxalase II, a representative of the metallo-β-lactamase superfamily, it could be shown that Zn(II) is essentially needed for the maintenance of the native structure of the enzyme and that its binding to the apoenzyme occurs during an important step of refolding [136]. For BcII the kinetics of metal ion binding have been studied by stopped-flow methods monitoring the metal ion-induced fluorescence changes of the protein. It could be shown that the association rate constants for binding of a second metal ion is two orders of magnitude lower compared to the first [54]. A rapid exchange of the zinc ion in the mononuclear enzyme was suggested to cause the slow association of a second metal ion and thus the negative cooperativity of zinc binding to BcII. The latter conclusion was based on the finding that Cd(II) in mononuclear BcII shows exchange between the two available binding sites on a microsecond time scale [113]. Theoretical calculations additionally support the idea of a flexible metal binding site in terms of strong dependence of the localization and distance of metal ions on the water structure around the active site [101].

A basic question concerns the physiological state of metallo-β-lactamases in their natural habitat. It is important to consider that all the enzymes studied so far were produced by overexpression in *Escherichia coli*. The purification is usually performed in the presence of high concentrations of zinc salts, and thus di-zinc enzymes are the end product of the production process. Additionally, it could be shown for some enzymes that the binuclear enzymes show higher activity towards various substrates. This led to the habit of numerous groups to study the enzymes always in the presence of excess zinc, which necessarily stabilizes the binuclear state. Therefore, almost all the inhibitor studies resulted in inhibition characteristics of the di-zinc enzymes. All the X-ray structures were obtained for the di-zinc form with the exception of the first BcII structure [11], which shows the mononuclear zinc enzyme. The latter fact could be attributed later to the very low pH value in the crystallization buffer [14]. Only for CphA, the enzyme from *A. hydrophila*, was it apparent that only the mono-zinc form might be physiologically relevant [37, 114]. When comparing the dissociation constants of zinc ions, however, it became apparent that formation of the binuclear zinc enzymes requires micromolar concentrations of free zinc for CphA [114] and BcII [54]. Already an early qualitative investigation with BcII from strain 5/B/6 of *Bacillus cereus* showed that the presence of nitrocefin as a substrate strongly reduced the dissociation constant of a first zinc ion bound [14]. This finding led to a systematic investigation of the influence of substrate presence on the zinc ion affinity of representatives from all three subclasses. The general finding was that the presence of substrates induces a decrease of the dissociation constant for a first metal ion bound to picomolar values, whereas the dissociation constant for a second metal ion generally increased such that a difference of five to six orders of magnitude between both macroscopic dissociation constants resulted [137]. The latter study concluded in a generally induced negative cooperativity of metal ion binding in the presence of imipenem as the substrate and suggested that the mono-zinc enzymes are the physiologically relevant species in the presence of substrates, whereas the metal-free state might dominate for some of the enzymes in the absence of substrates. It could be shown that formation of binuclear zinc enzymes generally required micromolar concentrations of free zinc in the presence of substrates [137]. In particular, the low affinity for a second zinc ion leads to the question whether or not physiological conditions offer sufficiently high metal ion concentrations to maintain the active metal-bound state of the enzymes. It was shown

that the metallothionein/thionein pair controls the available concentration of free zinc ions in eukaryotic cells [138]. From the presence of free thionein in different cell types combined with the picomolar dissociation constant of metallothionein [139], it could be concluded that free zinc ion concentrations in eukaryotic cells might be in the picomolar range [137]. Based on the study of metalloregulatory proteins, femtomolar concentrations of free cytosolic zinc were estimated in bacteria [140]. Although the relevant free zinc ion concentrations in infected foci cannot be found in the literature, they are expected to be extremely low. Since very low concentrations of free zinc might be assumed for the operational area of metallo-β-lactamases (periplasm or the extracellular environment of bacteria), physiological conditions might therefore not fulfil the requirements for a metal-bound enzyme state. It has been concluded that, due to the very low concentrations of free zinc ions in cellular environments, metallo-β-lactamases are most likely in the apo-enzyme state in the absence of substrates and that substrate availability might induce a spontaneous self-activation at picomolar free zinc ion concentrations, resulting in formation of active mononuclear zinc enzymes. The binuclear state was considered to be stabilized only at unphysiologically high zinc concentrations and might therefore be considered an artificial state [137]. Due to the fact that the two dissociation constants for binding of zinc ions to e. g. L1 are very similar, it is impossible to produce a homogeneous mono-zinc L1 in the absence of substrates. This led others to conclude that only the binuclear enzyme might be relevant for catalysis [71]. When following the kinetics of imipenem cleavage by Zn_2-L1 without added free zinc, and adding fresh imipenem several times, it appeared that the observed catalytic activity drops after several cycles [141] to activities obtained for the Zn_1-L1 [137]. Meanwhile, it has been shown that IMP-1, the enzyme from *Pseudomonas aeruginosa*, is also active as the mono-zinc enzyme [142], which is also supported by a pH dependence study of metal ion binding and activity [143]. All the metallo-β-lactamases tested so far are active as the mononuclear enzymes. Nevertheless, all of them, including CphA, have two metal ion binding sites. It appears extremely unlikely that nature has conserved such a structural motif if it is not functional. Starting from the assumption that the mononuclear enzymes might be the functional entities under physiological conditions, the role of the binuclear binding site during catalysis remains to be determined. The observed dynamics of mononuclear sites might already hint at a possible translocation of the metal ion during catalysis, but until now there is no experimental evidence directly demonstrating such transients except the cryo-spectroscopic data on Co(II)-substituted BcII [22, 23].

Until now, all inhibition studies published used excess zinc in the assays and thus forced the enzymes into a binuclear state. Whether this approach results in data which can be transferred to physiological conditions remains to be shown. If, however, the mononuclear enzyme is the predominating species at low zinc concentrations, it should be the target for inhibition studies. The suggestion that in the absence of substrates the enzymes might even exist as the metal-free species [137] leaves the open question whether the apo-enzymes might even be relevant targets for inhibitor design. Compounds which were able to prevent metal ion binding might serve as very potent inhibitors.

Some of the most interesting questions are not yet answered. Do the metallo-β-lactamases use mono- and binuclear mechanisms alternatively to fight different β-lactams? Do the mononuclear enzymes use different binding modes for different substrates? Or, does a single zinc ion use both binding sites during a catalytic cycle?

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