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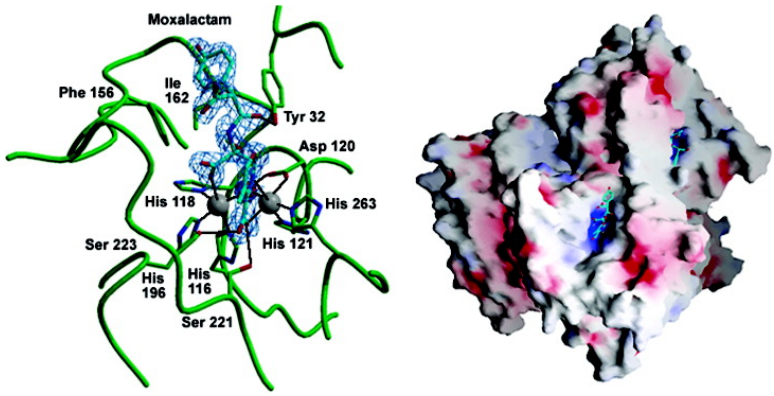
J. Am. Chem. Soc., **2005**, 127 (41), 14439-14444• DOI: 10.1021/ja0536062 • Publication Date (Web): 27 September 2005



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Antibiotic Recognition by Binuclear Metallo-β-Lactamases Revealed by X-ray Crystallography[#]

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Abstract: Metallo-β-lactamases are zinc-dependent enzymes responsible for resistance to β-lactam antibiotics in a variety of host bacteria, usually Gram-negative species that act as opportunist pathogens. They hydrolyze all classes of β-lactam antibiotics, including carbapenems, and escape the action of available β-lactamase inhibitors. Efforts to develop effective inhibitors have been hampered by the lack of structural information regarding how these enzymes recognize and turn over β-lactam substrates. We report here the crystal structure of the *Stenotrophomonas maltophilia* L1 enzyme in complex with the hydrolysis product of the 7α-methoxyoxacephem, moxalactam. The on-enzyme complex is a 3′-*exo*-methylene species generated by elimination of the 1-methyltetrazolyl-5-thiolate anion from the 3′-methyl group. Moxalactam binding to L1 involves direct interaction of the two active site zinc ions with the β-lactam amide and C4 carboxylate, groups that are common to all β-lactam substrates. The 7β-[(4-hydroxyphenyl)malonyl]-amino substituent makes limited hydrophobic and hydrogen bonding contacts with the active site groove. The mode of binding provides strong evidence that a water molecule situated between the two metal ions is the most likely nucleophile in the hydrolytic reaction. These data suggest a reaction mechanism for metallo-β-lactamases in which both metal ions contribute to catalysis by activating the bridging water/hydroxide nucleophile, polarizing the substrate amide bond for attack and stabilizing anionic nitrogen intermediates. The structure illustrates how a binuclear zinc site confers upon metallo-β-lactamases the ability both to recognize and efficiently hydrolyze a wide variety of β-lactam substrates.

Introduction

Metallo- or class B β-lactamases (mβls) present a challenge of increasing significance to the clinical effectiveness of β-lactam antibiotics. Since their initial discovery in clinically innocuous *Bacillus cereus* strains,[1] more than fifteen distinct enzymes have been identified in a range of Gram-negative bacterial species including several (*Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Bacteroides fragilis*, *Stenotrophomonas maltophilia*) that are opportunist pathogens of immunocompromised individuals such as transplant, chemotherapy and HIV patients.[2] While the earliest discovered mβls were chromosomal, several have now been mobilized on both plasmids and integrons and may thus be more readily disseminated through the microbial community in response to therapeutic pressure.[3] Although these enzymes can efficiently hydrolyze a wide variety of β-lactam substrates of all the main classes (penicillins, cephalosporins, and carbapenems) it is their activity against carbapenems that is of most immediate concern.[4] Carbapenems, the newest and most powerful generation of β-lactams, escape the activity of all but a few of the more widespread active-site serine β-lactamases. They are of particular value in the case of infection by species, such as those above, where the combination of an inherent lack of susceptibility to some classes of antibiotics and acquired genes that confer resistance to previously effective agents can severely limit the range of treatment options.[5] To date, there is no clinically useful mβl inhibitor.

Crystal structures have been published for a total of seven mβls,[6−12] all of which adopt an αβ/βα sandwich fold, so far

[#] Coordinates have been deposited with the protein data bank with accession number 2AIO.

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10.1021/ja0536062 CCC: $30.25

unique to the mβl superfamily, in which the catalytic zinc-binding site lies at the interface between the two domains. In the majority of structures this is a binuclear metal site with closely spaced (3.6−3.7 Å) tetrahedrally (Zn1) and trigonally bipyramidally (Zn2) coordinated zinc ions "bridged" by a water/hydroxide ion in an arrangement superficially reminiscent of the active sites of several binuclear metallohydrolyases.[13−15] In addition to the "bridging" water, Zn1 is ligated by three conserved histidine residues and Zn2 by a second "apical" water, an invariant aspartate, another histidine and either a cysteine or a second histidine residue. These, and accumulating theoretical[16,17] and mechanistic data,[18−21] have informed numerous models for the binding and subsequent hydrolysis of β-lactam substrates by binuclear mβls. However, detailed understanding of mβl mechanism has been hindered by the lack of structural information on the nature of the interactions between mβls and β-lactams.

A number of lines of evidence highlight the importance of interactions of the metal ions with substrate for both binding and turnover. (1) The *B. cereus* BcII apo-enzyme is unable to bind substrate.[22] (2) Visible absorbance and magnetic circular dichroism spectra of Co^{2+}-substituted BcII indicate changes in coordination of bound metal ion(s) during the catalytic cycle.[23] (3) Recent data from freeze-quench EPR studies of the Co^{2+}-substituted *S. maltophilia* L1 enzyme confirm that significant changes in the environment of at least one metal ion occur on binding and reaction with penicillin, cephalosporin, or carbapenem substrates.[21] (4) Interaction of an unprotonated nitrogen with Zn2 has been suggested to stabilize a ring-opened nitrogen anion intermediate whose existence is inferred from spectroscopic studies of the hydrolysis of the cephalosporin nitrocefin by several mβls.[18] (5) In the mononuclear enzyme CphA, from *Aeromonas hydrophila*, a crystallographic complex with the carbapenem biapenem reveals contacts of both the β-lactam nitrogen atom and the carboxylate group at C3/C4 with Zn^{2+}.[12]

Specific protein residues have also been suggested to contribute to the recognition and hydrolysis of β-lactams by mβls. The well-conserved lysine-224 (standard BBL numbering[24]) may interact with the C3/C4 carboxylate while asparagine-233 could, with Zn1, form an oxyanion hole to stabilize the tetrahedral oxyanionic species formed by attack of the "bridging" water/hydroxide on the carbonyl carbon. While structures of inhibitor complexes to some extent support these models,[9,25−27] mutagenic studies[28−30] suggest that the magnitude of these effects varies widely between classes of substrates (cephamycins, penicillins, cephalosporins etc.) and is not consistent between different enzymes. Conservation of these two residues is also incomplete across the range of enzymes and subclasses.[24] Similarly, although flexible loop regions adjacent to the mβl active site, for example residues 60−66 in *P. aeruginosa* IMP-1 or 156−162 in L1, do contribute to both binding and turnover of substrates, these effects vary for different enzymes and their magnitude is dependent upon the particular substrate.[31−33]

Taken as a whole these results suggest an active site tolerant to amino acid substitutions and able to accommodate a large array of alternatively substituted β-lactams. It is therefore an attractive notion that this broad substrate specificity arises from a mode of binding that optimizes contacts between common components of the β-lactam scaffold and the binuclear metal center.[8] Design of inhibitors for such a system presents a formidable challenge that is exacerbated by the paucity of structural information regarding mβl:β-lactam complexes. To date the only crystallographic information regarding the interaction of mβls with β-lactams is derived from the atypical mononuclear CphA enzyme that functions as a strict carbapenemase.[12]

As part of an ongoing investigation into the structure, mechanism and inhibition of mβls[8,20,34] we have therefore used the L1 enzyme of *S. maltophilia*, a significant hospital-acquired pathogen responsible for respiratory and urinary tract infections, bacteremias and endocarditis,[2] in soaking experiments to isolate β-lactam complexes for structure determination. We report here the 1.7 Å resolution crystal structure of L1 in complex with the hydrolyzed form of the oxacephem antibiotic, moxalactam.

Materials and Methods

Native *S. maltophilia* L1 was overexpressed, purified, and crystallized as previously described.[8,35] Moxalactam (Sigma, mixed stereoisomers) was dissolved in cryobuffer (100 mM HEPES pH 7.5, 2 M ammonium sulfate 1.5% PEG400, 25% glycerol) at concentrations between 10 and 100 mM and L1 crystals transferred directly into this solution for soaking times ranging from 45 s to 165 min. Crystals were retrieved into rayon loops (Hampton Research) and frozen by rapid plunging

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Table 1. Data Processing and Refinement Statistics[a]

Data Collection	
beamline	SRS 14.2
wavelength	0.978 Å
space group	$P6_422$ $a = b = 105.02$ Å, $c = 98.23$ Å
resolution	30−1.7 Å
R_{merge}	0.057 (0.246)
total reflections	145 544
unique reflections	33 459
$I/\sigma(I)$	24 (2.5)
completeness	93.6% (78.6%)
redundancy	4.3 (2.0)
Refinement	
$R_{working}$	17.1%
R_{free}	20.0%
Rmsd (bond lengths)	0.011 Å
Rmsd (bond angles)	1.28°
no. protein atoms	2001
no. water molecules	330

[a] Figures in parentheses refer to statistics for highest resolution shell (1.78−1.70 Å). R_{free} was calculated with 5% of data excluded from refinement.

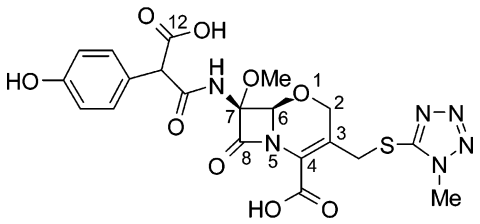

Figure 1. Structure of moxalactam.

into liquid nitrogen. All crystal manipulations were carried out at 4 °C. Data were collected on stations 14.1 and 14.2 of the UK synchrotron radiation source, Daresbury using a Quantum 4 CCD detector (Area Detector Systems Corporation, Poway, California, USA). Diffraction intensities were integrated, scaled and merged using DENZO/SCALEPACK.[36] Structures were solved by molecular replacement with AMORE[37] using the unliganded L1 monomer structure 1sml[8] as a search model and refined with REFMAC 5.0[38] using ARP/wARP version 5.0[39] to add crystallographic water molecules. Data collection and refinement statistics are presented in Table 1.

For mass spectrometry L1 at 35 μM in 50 mM sodium cacodylate 100 mM NaCl pH 7.0 was added to dry moxalactam powder to a final concentration of 100 mM, incubated overnight at room temperature and the enzyme removed by passage through a 10 kDa cutoff MicroCon spin filter (Amicon). The resulting yellow solution was diluted in water and injected onto the electrospray instrument (Applied Biosystems) fitted with an in line C4 column.

Results

Direct addition of moxalactam (Figure 1) to native L1 crystals resulted in only minor perturbation of the crystallographic lattice, as adjudged by the unit cell parameters and quality of diffraction data (Table 1) compared with native crystals.[8] Diffraction data extending to 1.7 Å Bragg spacing yielded high quality electron density maps (Figure 2a) that enabled us to build hydrolyzed moxalactam product and 330 water molecules (see relevant crystallographic statistics in Table 1). The quality of the final model gives us strong confidence in the geometry of the interactions between the protein, metal ions and ligand.

Electron density for the substrate oxacephem nucleus was clearly visible in the active site after soaking for 225 s in 100 mM moxalactam. However, extended soak times (>20 min) in 10 mM moxalactam were required to resolve the 7β substituent group. A difference Fourier map using data from a 90 min soak is shown in Figure 2(a). The β-lactam ring is significantly distorted away from square planar geometry with a 2.75 Å separation between the β-lactam carbonyl carbon and nitrogen atoms and a clear discontinuity in the density between them. Electron density at the β-lactam carbon reveals a carboxylate rather than carbonyl group. The snap-frozen crystalline state thus corresponds to a hydrolyzed form of the antibiotic. Similar electron density at this carbonyl carbon was observed at all time points for which interpretable maps were obtained.

The opened β-lactam ring, the oxacephem ring and both the 7α-methoxy and 7β-[(4-hydroxyphenyl)malonyl]-amino substituent groups are well defined in experimental electron density maps (Figure 2a). However, no density at any time point could be observed for the 1-methyl-5-thiotetrazole leaving group. Although the chemical instability of moxalactam prevented us from obtaining mass spectrometric data directly from the crystalline complex, the mass of the major product of L1-catalyzed hydrolysis of moxalactam in solution is within 0.3 Da of that calculated for the observed 3′-*exo*-methylene species.

Hydrolyzed moxalactam binds to the enzyme through interactions with both zinc ions and with protein side chains adjacent to the L1 active site (Figure 2b−d). These interactions mainly involve portions of the moxalactam molecule that are common to most β-lactams (amide nitrogen and carbonyl groups and C4 carboxylate) and are thus likely to be replicated in complexes with a wide range of antibiotics. At Zn1 (tetrahedral in the unliganded structure) one oxygen of the product C8 carboxylate acts as an additional ligand, thus increasing coordination to 5-fold in a distorted trigonal bipyramidal geometry. At Zn2 the carboxylate at moxalactam C4 displaces the "apical" water molecule present in the unliganded complex while the β-lactam nitrogen atom (N5) approaches from a direction opposite to the His-121:Zn^{2+} coordination bond to complete an octahedral coordination geometry. Details of the metal:ligand distances for both zinc ions are given in Table 2. In addition to the protein ligands (His-116, -118, -121, -196, and -263, and Asp-120) coordination of the two metal ions is completed by a water molecule positioned between them in a "bridging" position. This water is positioned within H-bonding distance of OD1 of Asp-120 (2.46 Å) and lies close to the ether oxygen atom of the 7α-methoxy group (2.91 Å).

Binding of the C4 carboxylate group is further facilitated by hydrogen bonding of both oxygen atoms to protein side chains. In the unliganded structure, the Zn2-bound "apical" water is positioned by a hydrogen bond with the side chain of Ser-221. This bond is preserved when the carboxylate oxygen substitutes for water. The second carboxylate oxygen points away from the metal site and contacts both the OG oxygen atom of Ser-223 and an adjacent solvent water molecule. The 7β substituent group is aligned along the shallow groove created at the interface of the two core β sheets (Figure 2c) such that the phenyl ring contacts a hydrophobic patch formed by the side chains of Phe-

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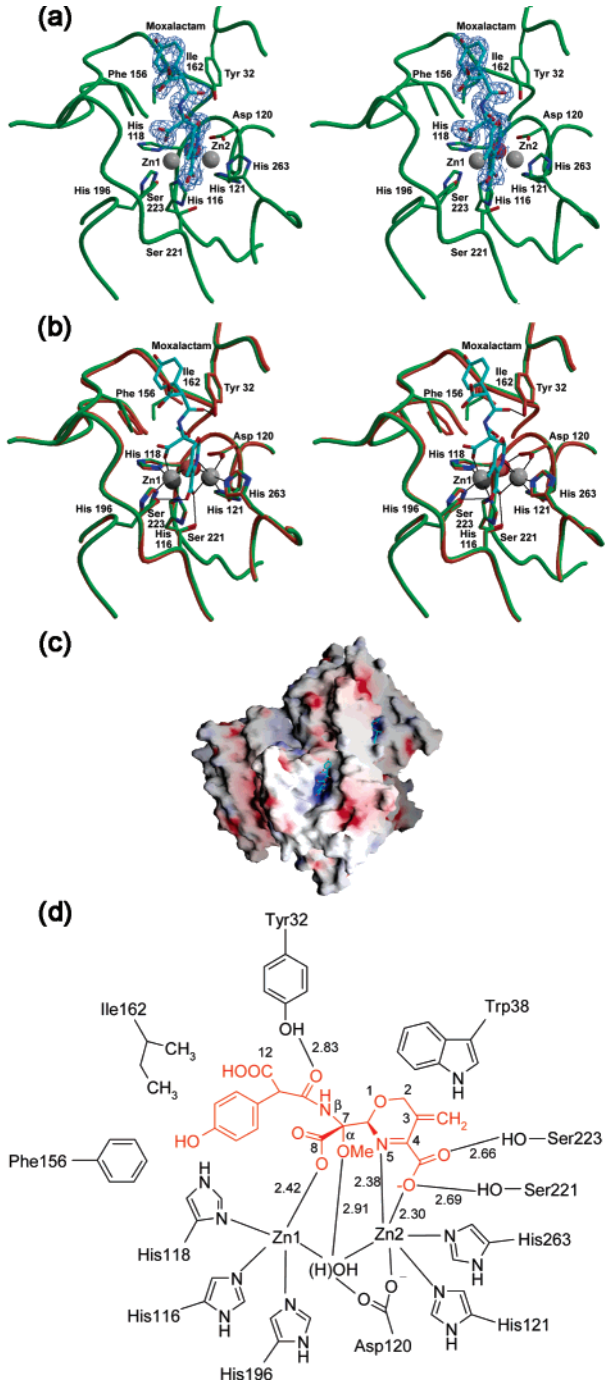

Figure 2. (a). Stereoview showing electron density for hydrolyzed moxalactam in the L1 active site. Map shown is $2F_{obs} - F_{calc}$ contoured at 1.5σ calculated with the ligand omitted from the model. Atom coloring is as standard with the exceptions that protein carbon atoms are in green and moxalactam carbons in cyan. Zinc ions are rendered as gray and the bridging water as a red sphere. This figure was prepared using MOLSCRIPT/BOBSCRIPT[40,41] and rendered with Raster3D.[42] Figure 2(b): Superposition of complex upon the unliganded L1 structure (pdb accession 1sml[8]). Complex structure colored as above; for the unliganded structure carbon atoms and the "bridging" water are shown in orange and zinc ions as dark gray spheres. Metal coordination and hydrogen-bonding interactions in the complex structure are shown as fine gray lines. Figure 2(c): Molecular surface representation of hydrolyzed moxalactam (shown as ball-and-stick) in the active site of the L1 tetramer. Zinc ions are rendered as gray spheres, moxalactam carbon atoms are shown in cyan. Surface is colored by electrostatic potential. This figure was prepared using the program GRASP.[43] Figure 2(d): Schematic showing interactions of hydrolyzed moxalactam (red) with the L1 active site. Distances (Å) given are between heteroatoms.

Table 2. Details of Zinc Co-ordination in Unligated (1sml,[8]) and Moxalactam Complex Structures[a]

Atom 1	Atom 2	distance (unliganded) (Å)	distance (moxalactam complex) (Å)
His 116 NE2	Zn1	2.03	2.20
His 118 ND1	Zn1	2.11	2.08
His 196 NE2	Zn1	2.05	2.07
Wat 1	Zn1	1.88	1.99
Asp 120 OD2	Zn2	2.07	2.19
His 121 NE2	Zn2	2.02	2.09
His 263 NE2	Zn2	2.07	2.10
Wat 1	Zn2	2.06	2.15
Wat 2	Zn2	2.40	
Asp 120 OD1	Wat1	2.83	2.46
Zn1	Zn2	3.46	3.68

[a] Additional interactions made in the complex alone are detailed in Figure 2d.

156, Ile-162, and Tyr-32 and the carbonyl oxygen hydrogen bonds to the hydroxyl of Tyr-32. Figure 2d provides a schematic overview of these interactions. A comparison with the unliganded structure[8] (Table 2, Figure 2b) shows that only minimal rearrangements of the active site are required to accommodate ligand. The most important differences relate to the position of the bridging water/hydroxide which in the complex moves 0.4 Å closer to Asp-120, 0.1 Å away from Zn2 and 0.1 Å away from Zn1. The Zn^{2+}:Zn^{2+} separation also increases by 0.2 Å. The Zn2:His-121 bond lengthens by 0.1 Å, consistent with the move to an octahedral geometry in which the water/hydroxide, Asp-120, His-263 and moxalactam nitrogen ligands are approximately coplanar.

Discussion

In our previous determination of the crystal structure of the unliganded L1 enzyme,[8] we used a manual docking and Monte Carlo minimization methodology to investigate the means by which mβls might recognize their substrates. Our present study directly reveals the interactions of the L1 mβl with a β-lactam substrate and provides important insights into the likely hydrolytic mechanism for binuclear mβls.

An important property of mβls is their comparative lack of selectivity for substrates.[44] They have the ability to hydrolyze many different classes of β-lactams (penicillins, cephalosporins, and carbapenems) within each class of which there is considerable variation in substituent groups at the C2/C3 and C6/C7 positions. Accordingly, we have previously proposed[8] that interactions of the two metal ions in the binuclear zinc site with invariant components of β-lactams, namely the amide carbonyl oxygen (C8) and nitrogen (N5) atoms and the C3/C4 carboxylate, provide the major contribution to substrate binding. In the current structure, we observe hydrolyzed moxalactam contacting both metal ions while interactions with protein are limited in comparison. Ligation of Zn1 by moxalactam C8 carboxylate (amide carbonyl in unhydrolyzed substrate) and Zn2 by N5 amide nitrogen and C4 carboxylate raises the coordination number of both metal ions while only marginally altering the geometry of the existing protein ligands (Figure 2b). Contact

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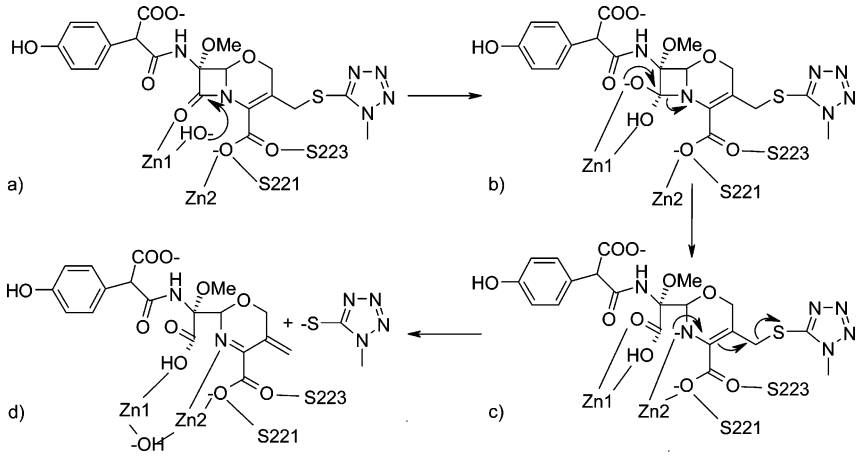
Scheme 1. Mechanism of Moxalactam Hydrolysis by the *S. maltophilia* L1 mβl.



of protein side chains with these invariant substrate moieties is limited to a bidentate interaction of C4 carboxylate with the serine residues at positions 221 and 223. Interestingly, the interaction with Ser-221 OG displaces the second "apical" Zn2-bound water. We propose that this protein:C4 carboxylate interaction is functionally conserved across the mβl enzyme family as evidenced by interactions involving the equivalent Lys-224 in other crystal structures.[9,25−27] Nevertheless, mutagenesis of Ser-223 in L1[45] or of Lys-224 in CcrA[28] and IMP-1[29] suggests that the effect on catalytic efficiency varies with substrate and in some cases is marginal. We thus consider the interaction of C3/C4 carboxylate with the metal ions to be of primary importance.

The extended 7α-(4-hydroxyphenyl)-malonyl moiety is readily accommodated in the open, shallow groove that is a feature of mβl active sites. With the sole exception of Tyr-32, interactions are limited to hydrophobic contacts with the Phe-156 to Ile-162 loop, a region we have previously predicted[8] to provide stabilizing interactions with substrates with more hydrophobic substituents at the 6 or 7 position. Nevertheless, mutation of this loop affects overall catalytic efficiency against β-lactams from all structural classes by no more than an order of magnitude.[45, 46]

The mode of binding that we describe provides insights into the hydrolytic mechanism of mβls. A catalytic mechanism incorporating these observations is shown in Scheme 1. It is widely believed that the "bridging" water/hydroxide is the most likely candidate to act as the reaction nucleophile in β-lactam hydrolysis by mβls.[7,8,18,47] In the present complex this argument is supported both by the observed displacement of the Zn2-bound "apical" water (considered the less likely of the two active site water molecules to fulfill this function[7]) by C4 carboxylate and by the overall orientation of moxalactam. The observed position of the bridging water/hydroxide ligand would enable it to make a *re*-face attack upon the carbonyl carbon of substrate bound with its amide carbonyl (C8) oxygen ligated to Zn1 and C4 carboxylate to Zn2. (Scheme 1a). The possibility remains that this may occur after a shift to a terminal position on Zn1, as has been proposed for other binuclear Zn^{2+}-hydrolases such as *Aeromonas* aminopeptidase.[48] Cleavage of the β-lactam amide bond, either by a mechanism concerted with nucleophilic attack to generate (c) directly from (a), or involving a tetrahedral oxyanion (b), and subsequent elimination of the 1-methyltetrazolyl-5-thiolate anion from the 3′-methyl group (c) would then generate the observed complex (d). However, although Zn2 and N5 are in close proximity (2.3 Å) the observed coordination reveals N5, C4, C6, and Zn2 to be some way from the coplanar geometry expected for the sp^2 imine nitrogen of the product. This arrangement would rather favor coordination by species, such as the ring-opened anionic intermediate observed in hydrolysis of nitrocefin by various di-zinc mβls,[18−20] where this nitrogen is sp^3. Progress along the reaction coordinate from intact substrate through the putative tetrahedral oxyanion (Scheme 1b) to a ring-opened nitrogen anion (Scheme 1c) will result in a progressive increase in the basicity of this nitrogen, improving its ability to ligate Zn^{2+}. The interaction of metal ions with the basic nitrogen of tetrahedral oxyanion species has been invoked to explain catalysis of penicillin and cephalosporin hydrolysis by metal ions in solution.[49] In the present structure, however, the close Zn2:N5 interaction that we observe appears to be possible only when cleavage of the β-lactam amide bond, and consequent rotation about the C6−C7 bond, permits N5 to move out of the plane of the β-lactam ring toward Zn2 (Scheme 1c; Figure 2a,b). This is in accordance with suggestions that ring opening of β-lactams may be achieved by a twisting motion, rather than stretching of the C−N bond as thought to occur in peptide hydrolysis reactions.[50,51] For unhydrolyzed substrate, maintaining close contact between N5 and Zn2 would require significant structural rearrangement within the active site.

We thus propose a dual role for the Zn2 ion in mβls. First, binding interactions of Zn2 with the β-lactam carboxylate contribute to the broad spectrum of activity of binuclear mβls. Second, Zn2 facilitates catalysis by requiring amide bond cleavage and rotation in the β-lactam ring to permit tight interaction with N5. We suggest therefore that the ring-opened nitrogen anion is the species best able to make this interaction.

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However, this may be short-lived except in cases such as nitrocefin where it is stabilized by additional factors such as a conjugated system within the 3′-dinitrostyryl moiety.[18] In the majority of cases, the steps preceding protonation of the anion, namely nucleophilic attack of hydroxide or cleavage of the β-lactam bond, will be rate-determining. Such a mechanism is attractive as it also reconciles observations made in kinetic studies of various enzyme:substrate systems[18,20,31] and enables the enzyme to offset the high energetic cost of breaking the β-lactam amide bond[50] by strengthening the nitrogen:Zn2 interaction in the ring-opened species. This proposal is also consistent with our previous suggestion,[8] based upon Monte Carlo simulations, that bound substrate may adopt a number of different conformations of approximately equivalent free energy. In the absence of the Zn2:N5 interaction no single, well-defined conformation for bound substrate may be favored. This may also explain why, after multiple attempts, we were unable to observe electron density corresponding to bound but unhydrolyzed substrate. For cephalosporins and oxacephems (such as moxalactam) with good 3′-leaving groups, formation of the imine product would occur by rearrangement of the anion and expulsion of the leaving group (Scheme 1d). Such a stepwise mechanism, requiring amide bond cleavage prior to leaving group elimination, is consistent with studies of oxacephem hydrolysis in free solution[52] and of cephalosporin hydrolysis by the *Staphylococcus aureus* PC1, *Enterobacter cloacae* P99, and RTEM-2 serine β-lactamases.[53, 54]

We consider two aspects of the observed structure as worthy of additional comment. Zn1 is coordinated by a single C8 oxygen of product rather than the bidentate, potentially μ-bridging, complex involving both oxygen atoms that might be expected to result from attack upon C8 of a bridging water/hydroxide nucleophile. In this respect, the present structure closely resembles that postulated for the complex of a dinuclear model compound with the nitrocefin reaction intermediate.[47] The observed complex could result if the carbonyl oxygen detached from Zn1 after attack of terminal Zn1-bound water/hydroxide,[28] although this conflicts with any possible role for Zn1 in stabilizing oxyanionic species (Scheme 1b).[51] Alternatively, displacement of carboxylate oxygen by an incoming water molecule at the bridging position could cause a rearrangement of coordination about Zn1 at a later stage in the reaction, as shown in Scheme 1. In this context, it is significant that the recent freeze-quench EPR study of β-lactam hydrolysis by Co^{2+}-L1 did not detect substrate-derived μ-bridging ligands for any of the four compounds studied.[21] Second, our structure may also explain the poor activity of L1 (low K_M and k_{cat}) against 7α-methoxy-substituted (cephamycin and oxacephem) substrates.[35] In relation to the unliganded enzyme (Figure 2b) the bridging water/hydroxide is displaced 0.4 Å closer to Asp-120 and may thus be removed from the optimal position for catalysis. The effect of the 7α-methoxy moiety on catalytic efficiency however varies significantly across the mβls and is far greater upon the B3 than the B1 subclass enzymes. This could reflect their differing active site geometries.[8]

Very recently[12] a structure (pdb accession 1 × 8i) was published revealing the interactions of the carbapenem biapenem with the mβl CphA from *Aeromonas hydrophila*, a single zinc enzyme with a strict specificity for carbapenem substrates. The hydrolyzed β-lactam is bound as an unusual fused bicylic structure presumably formed after amide bond hydrolysis. Ligand binding is mediated by interaction of the single zinc ion (occupying the Zn2 position) with the C4 carboxylate oxygen and with the β-lactam nitrogen N5. However, the proposed catalytic mechanism utilizes histidine-118 to polarize and orient the water nucleophile and bears little resemblance to what we propose here. Moreover, although the suggestion has been made that under physiological conditions all mβls function with a single zinc ion bound,[55] our data highlight the importance of both metal ions in the binuclear zinc site for both substrate binding and catalysis. Our structure suggests that Zn1 binds substrate via the β-lactam carbonyl oxygen and contributes to catalysis by polarizing this bond and by activating the water nucleophile. Zn2 acts to recognize the invariant C4 carboxylate of β-lactams, may also activate the bridging water nucleophile and, by acting as a Lewis acid to stabilize an N5 nitrogen anion, promotes cleavage by rotation of the β-lactam amide bond. We suggest that possession of a binuclear metal site thus improves catalytic efficiency and may be the key to these enzymes' unusual lack of selectivity by enabling them to maximize productive binding interactions with a limited subset of key functionals. This lack of discrimination with respect to substrate is manifest clinically as broad-spectrum β-lactam resistance in pathogenic hosts. Our structure provides the first direct identification of the determinants for substrate recognition by binuclear mβls and as such will facilitate the rational design both of new β-lactams with reduced susceptibility to mβls and of non-β-lactam mβl inhibitors.

Acknowledgment. J.S. is a Beit Memorial Fellow for Medical Research. We thank James Nicholson, Pierre Rizakallah, Phil Walker, and Julie Clapperton for assistance with data collection and Michael Crowder, John Buynak, and attendees at the 8th European β-lactamase workshop for useful discussions.

Supporting Information Available: Complete citations for refs 9, 26, 27. This material is available free of charge via the Internet at http://pubs.acs.org.

JA0536062

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