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A minimalistic approach to identify substrate binding features in B1 Metallo-β-lactamases

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Abstract—The 2-oxoazetidinylacetate sodium salt was synthesized as a model of a minimal β-lactam drug. This compound and the monobactam aztreonam were assayed as substrates of the Metallo-β-lactamase BcII. None of them was hydrolyzed by the enzyme. While the azetidinone was not able to bind BcII, aztreonam was shown to bind in a nonproductive mode. These results provide an explanation for the unability of Metallo-β-lactamases to inactive monobactams and give some clues for inhibitor design.

Metallo-β-lactamases (MβLs) are zinc enzymes that hydrolyze β-lactam antibiotics, representing one of the main resistance mechanisms developed by bacteria toward antibiotics.[1] These enzymes differ from the well-known serine-β-lactamases in that they display a broad substrate range, being capable of inactivating penicillins (**1**), carbapenems (**2**), and cefalosporins (**3**) (Fig. 1).[2] Despite sharing a conserved fold, MβLs are quite diverse in active site structure, and (possibly) catalytic mechanisms.[3,4] MβLs can bind up to two zinc ions, which are essential for substrate binding and hydrolysis.[5] The broad structural variation among substrates and enzymes has impeded the identification of common structural determinants of substrate recognition, thus thwarting inhibitor design.[6]

MβL substrates share the β-lactam ring and a carboxylate moiety α to the β-lactam nitrogen, which we tentatively define as the 'minimal β-lactam substrate'. This carboxylate group is supposed to interact with Zn2 and a positively charged residue in the active site, while Zn1 delivers the attacking nucleophile.[7,8] On the other hand, monobactams, such as aztreonam (**4**), are the only family of β-lactam antibiotics that are not efficiently hydrolyzed by MβLs. So far, the reasons that make

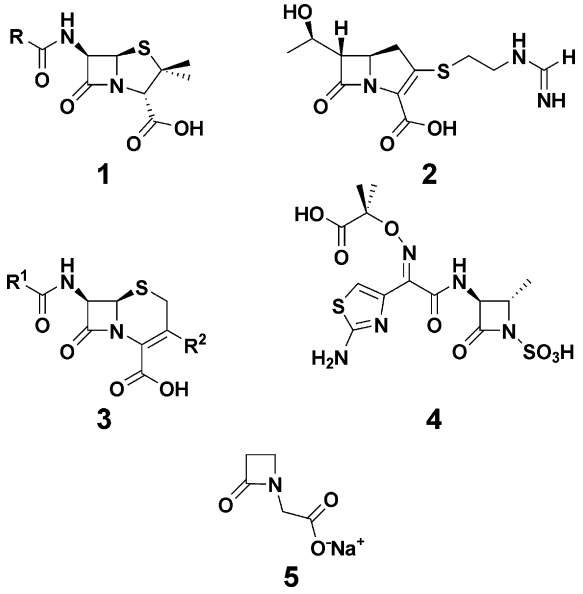

Figure 1. Representative examples for the most common β-lactam antibiotic families. Penicillin (**1**), carbapenem (**2**), cefalosporin (**3**), and monobactam (**4**), and 2-oxo-azetidin-1-yl-acetate sodium salt (**5**), the proposed 'minimal β-lactam substrate' structure.

Keywords: Metallo-β-lactamases; Inhibitor design; Ligand binding; Monocyclic β-lactams.
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aztreonam resistant to MβL-mediated hydrolysis are not known.[3] Here we decided to test whether aztreonam is able to bind MβLs, and in so doing, to compare its behavior with that of the model azetidinone compound (**5**), representing the 'minimal β-lactam substrate'.

Substrate hydrolysis. Hydrolysis of aztreonam (**4**) and the azetidinone (**5**) by the MβL BcII was studied by UV spectroscopy.[9] None of these compounds showed a noticeable hydrolysis over a long time (16 h), even when adding high enzyme concentrations (10–20 μM). The absence of hydrolysis was also verified by ^{1}H NMR spectroscopy. These assays reveal that neither aztreonam nor azetidinone (**5**) are suitable substrates of BcII. This may be attributed to either lack of binding of these compounds at all to the enzyme, or to a nonproductive binding mode. Since the finding of a binding, nonhydrolyzable compound could be exploited to design a possible inhibitor, we explored binding by different biophysical methods.

Binding to Co(II)-BcII monitored by UV–vis and fluorescence spectroscopy. Co(II)-substitution has been largely exploited in zinc enzymes as a probe of the metal site structure, and to follow changes upon exogenous ligand binding. The spectral features of Co(II)-BcII have been already characterized in detail.[10] The spectrum is composed of ligand-field bands in the visible region attributed to the Co1 site and stronger absorption bands from the Co2 site in the UV region due to ligand → metal charge transfer. Addition of azetidinone (**5**) did not induce spectral changes in the absorption spectrum of Co(II)-BcII. A steady decrease in the intensity of all bands was observed at high concentrations of added azetidinone (**5**), suggesting metal ion dissociation. When aztreonam was added, no changes could be observed in the visible features of the spectrum. The charge transfer band of Co(II) BcII at 343 nm was obscured by an intense band of aztreonam in this range, and could not be followed during this titration.

Ligand binding to BcII can also be followed by conformational changes in the enzyme that induce variations in the intrinsic Trp fluorescence.[11] No changes in the fluorescence intensity could be monitored upon addition of azetidinone (**5**) in a stopped flow instrument. Moreover, the addition of azetidinone (**5**) was unable to modify nitrocefin binding parameters. In the case of aztreonam, the absorption of this compound limited the detection of this phenomenon, suggesting that it binds very weakly or does not bind at all.

Binding to BcII monitored by NMR spectroscopy.[12] Finally, we employed NMR spectroscopy to follow ligand binding by chemical shift perturbation (CSP) of the backbone amide residues of BcII.[13] Consequently, ^{15}N-BcII was expressed in *Escherichia coli* BL21(DE3) *pLysS*' cells transformed with the pET-Term-BcII plasmid.[10] The corresponding ^{15}N, ^{1}H HSQC spectra were recorded upon additions of increasing concentrations of **4** and **5**. The HSQC spectrum displayed no changes when azetidinone (**5**) was added up to 100 mM final concentration, revealing no binding at all, in agreement with the UV–vis and fluorescence studies.

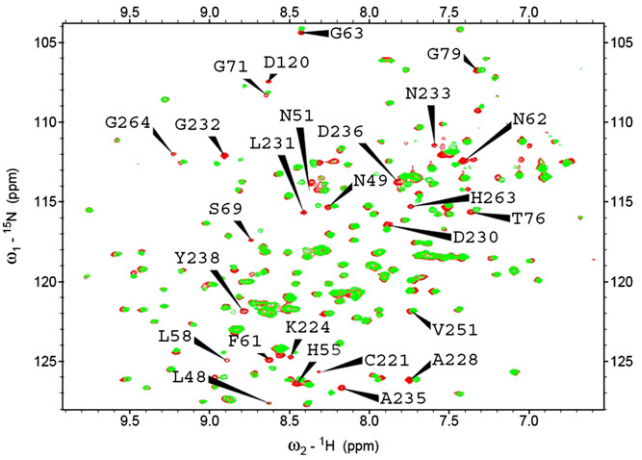

Figure 2. Corresponding ^{15}N,^{1}H HSQC spectra for free BcII (red) and BcII plus 40 mM aztreonam (green). The signal labeled as N233 is assigned to the NH sidechain of the corresponding residue.The NMR data processing and graphic rendering was done with Sparky (T. D. Goddard and D. G. Kneller, *SPARKY 3*, *University of California, San Francisco*).

Addition of aztreonam (**4**) induced spectral changes at very high concentrations (>40 mM) (Fig. 2), that are summarized in Table 1. These changes are small but significant. The nature of the resulting shifts indicates a fast exchange regime, typical of weak ligand binding. The perturbed residues include the three Zn2 ligands (D120, C221, H263), K224 (that usually interacts with the carboxylate moiety of bicyclic β-lactams), S69 (a second-shell ligand of the Zn2 site, which is involved in a hydrogen bond with D120), and residues belonging to loops 3 and 12, that flank the active site.[7] The behavior of the loops resembles that observed upon inhibitor binding for the enzyme CcrA,[14] which is another B1 subclass partner of BcII. In contrast to the situation of CcrA, there is a neat discrimination between the two zinc binding sites, since Zn1 ligands appear largely nonperturbed while Zn2 is involved on aztreonam binding.

We docked aztreonam in the active site of BcII based on the CSP map and positioning the sulfonate moiety on the Zn2 coordination sphere lying away from Zn1 site.[15] As a result, the β-lactam moiety is at ~5.5 Å from the attacking nucleophile at the Zn1 site and in an improper orientation, in a nonproductive binding mode. The NMR data fully agree with the Co(II) substitution experiments, that show no interaction of the ligand with the Co1 site (as results from the absence of changes in the absorption features in the visible range). Thus, we can conclude that aztreonam binds poorly and in a nonproductive way to BcII. The same result can be extrapolated to all B1 MβLs. Since aztreonam was designed with 4-methyl substituent to escape the hydrolysis by serine-β-lactamases, we cannot completely discard that this moiety is also effective in this case.

Table 1. Most significant chemical shift perturbations on BcII residues upon addition of aztreonam (**4**)

Residue[a]	CSP (ppm)	Location[b]
N233[c]	0.64	Loop 12
S69	0.61	S4, Zn2 second-shell ligand
F61	0.53	Loop 3
G232	0.51	Loop 12
L58	0.51	S3
N51	0.51	S3
L231	0.44	Loop 12
H263	0.39	S12, Zn2 ligand
Y238	0.33	Loop 12
H55	0.33	S3
D120	0.31	Zn2 ligand
A235	0.29	Loop 12
D230	0.29	Loop 12
K224	0.29	Loop 12
G63	0.26	Loop 3
C221	0.20	Zn2 ligand
H116	0.10	Zn1 ligand
H118	0.04	Zn1 ligand
H196	0.00	Zn1 ligand

The three His ligands of the Zn1 site are included for comparison.
[a] BBL numbering.
[b] S, β-strand; Zn1, zinc 1 coordination site; Zn2, zinc 2 coordination site, for loops' numbering, see Dal Peraro et al.[7]
[c] The perturbed signal corresponds to the Nδ2–Hδ21 of residue N233.

Regarding azetidinone (**5**), the 'minimal β-lactam substrate', it is striking that this compound is not able to bind BcII. This may be attributed to the mobility of the carboxylate moiety when the second ring is not present, or to the absence of other substituents that (not being conserved) may provide additional nonspecific anchoring points in the enzyme structure. Analyzing K_M from several substrates it is clear that substrates without a substituent chain in position 6 of the penicillanic nucleus, or position 7 of the cephalosporanic nucleus, have the highest K_M values.[2,16] Overall, this suggests that the disparate substituents in this position help in providing nonspecific, hydrophobic anchoring points to β-lactam substrates. Given that azetidinone (**5**) and aztreonam (**4**) are both susceptible to alkaline hydrolysis, the lack of an efficient binding mode is the principal factor for the absence of enzymatic hydrolysis for both compounds.

Here we show that the minimal β-lactam substrate is larger than expected, suggesting that some seemingly ancillary, hydrophobic, substituents may play a role in assisting substrate binding. This should be taken into account for future inhibitor design efforts.

Acknowledgments

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Supplementary data

Supporting information is available with detailed procedures for the synthesis of azetidinone (**5**) and the spectroscopic data of azetidinone (**5**) and the synthetic precursors. Also, UV–vis spectroscopy data of Co(II) substituted BcII titration with aztreonam and azetidinone (**5**) are included. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.06.089.

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12. For NMR experiments, labeled BcII was obtained as follows: an 8 h LB (150 μg/mL ampicillin and 35 μg/mL cloramphenicol) culture of the transformed cells was pelleted and inoculated into a 100 mL M9 medium supplemented with 4 g/L glucose, 1.2 g/L $(NH_4)_2SO_4$, 1 mM $MgSO_4$, 10 μM $CaCl_2$, 150 μg/mL ampicillin, and

35 μg/mL cloramphenicol. Cells were grown at 37 °C overnight, pelleted, and resuspended in 2 L of the same fresh medium, but ^{15}N $(NH_4)_2SO_4$ was used. Cells were grown for 4–5 h at 37 °C until OD_{600nm} = 0.7 was reached. Expression was induced by adding 1 mM isopropyl-thiogalactoside and 100 μM $ZnSO_4$. Cells were cultured for additional 4–5 h until saturation (OD_{600nm} = 1.8) was reached. Cells were harvested and BcII was purified as previously reported by Orellano et al. NMR experiments were carried out at 308 K in Avance II 600 Bruker spectrometer using buffer MES 100 mM, NaCl 200 mM, $ZnSO_4$ 200 μM, pH 6.4. BcII samples were in the 0.25–1.1 mM concentration range. Azetidinone (**5**) and aztreonam (**4**) stock solutions were 1 M in the same buffer but without added zinc(II).

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