Role of a solvent-exposed tryptophan in the recognition and binding of antibiotic substrates for a metallo- β -lactamase

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Abstract

Numerous X-ray crystal structures of the metallo- β -lactamase from *Bacteroides fragilis* and related organisms show a β -hairpin loop immediately adjacent to the active-site zinc atom(s). Both crystallographic and NMR information show that the end of this β -hairpin loop, which contains a solvent exposed tryptophan residue, Trp49, is highly flexible in the absence of substrates or other ligands, giving rise in some of the X-ray structures to a lack of observable electron density in this region. We report an investigation of the role of this mobile, solvent-exposed tryptophan using site-directed mutagenesis, steady state kinetics measurements and characterization by NMR. Trp49 appears to have a role both in substrate binding and in promotion of catalysis. Substitution of this residue with a number of different side chains indicates that the binding interaction depends on the bulky hydrophobic and aromatic nature of the indole ring, which can provide relatively non-specific interactions with a variety of antibiotic substrates. In this way, the tryptophan at this position provides a large degree of the breadth of substrate specificity for the metallo- β -lactamase. Previous studies established that the antibiotic binding site was sufficiently plastic that the derivatization of existing antibiotics is unlikely to result in the successful treatment of bacterial infections incorporating this resistance element. Rather, a more productive approach may be to design therapeutics directed towards this solvent-exposed tryptophan residue.

Keywords: Lactamase; mutant; enzyme kinetics; lactamase inhibition

Over the last decade, the appearance of pathogenic bacteria resistant to commonly used antibiotics has become a growing public health concern (Hughes and Tenover 1997; Normark and Normark 2002; Sandanayaka and Prashad 2002). While steady advances have been made in the understanding of the general nature of antibiotic resistance, it continues to be a problem for clinicians in the treatment of disease associated with antibiotic-resistant organisms. Therefore, investigations to better characterize the precise mechanisms of resistance are essential for the continued development of

novel therapeutic strategies and more effective therapeutic agents.

While several distinct cellular mechanisms of antibiotic resistance have been characterized (Livermore 1998; Matagne et al. 1999), the most common cause is through bacterial enzymes termed β -lactamases that recognize and degrade popular β -lactam antibiotics. Four major classes of β -lactamases have been delineated and grouped based upon amino-acid sequence similarities (Bush 1989a,b; Bush et al. 1995). Classes 1, 2, and 4 are serine hydrolases and can be inhibited by compounds such as sulbactam and clavulanic acid (Bush 1989a). Group 3 enzymes, which include all of the metal-requiring (metallo) β -lactamases, have been shown to hydrolyze most β -lactam antibiotics, but have shown little susceptibility to currently used β -lactamase inhibitors.

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Several organisms have been shown to produce metallo- β -lactamases, including *Klebsiella* (Senda et al. 1996), *Stenotrophomonas maltophilia* (Ullah et al. 1998), *Serratia marcescens* (Yang et al. 1990), *Bacillus cereus* (Sabath and Abraham 1966), and *Bacteroides fragilis* (Cuchural et al. 1986). Metallo- β -lactamases are generally monomeric, except for the enzyme from *Stenotrophomonas maltophilia*, which is tetrameric. The metallo- β -lactamase from *Bacteroides fragilis* is one of the most problematic of lactamases characterized thus far, mainly due to its high turnover and broad specificity against a wide variety of antibiotic substrates (Carfi et al. 1998). A standard numbering scheme for metallo- β -lactamases has recently been published (Galleni et al. 2001).

The overall structure of the 25 kD metallo- β -lactamase from *Bacteroides fragilis* has been shown to consist of a four-layered $\alpha\beta\alpha$ motif, and is representative of the metallo- β -lactamase superfamily (Fig. 1; Concha et al. 1996; Carfi et al. 1998; Ullah et al. 1998). The enzyme active site is present at one end of the β -sandwich and contains a binuclear zinc site. One zinc ion is coordinated by three histidine residues (His99 N ϵ , His101 N δ , and His162 N ϵ) and a single water molecule. The second ion is coordinated by three residues (Asp103 O δ 2, Cys181 S γ , and His223 N ϵ) and two water molecules. One water molecule is coordinated (bridged) by both zinc ions and is thought to exist in a hydroxide form (Wang and Benkovic 1998; Wang et al. 1998). Binding of a lactam substrate is followed by hydroxide attack, a reaction facilitated by Asn193, presum-

ably through formation of an oxy-anion hole (Yanchak et al. 2000). Collapse of the tetrahedral adduct can expel the lactam nitrogen without protonation, leading to an anionic intermediate, detected when using nitrocefin (Fig. 2) as a substrate (Wang et al. 1998). Decay of this intermediate to product occurs through a rate-limiting protonation event (Wang et al. 1999; Yanchak et al. 2000). However, less activated substrates may require protonation of the leaving lactam nitrogen before cleavage (Spencer et al. 2001).

Flanking the enzyme active site at the edge of the β -sandwich are two extended loop regions (Fig. 1). The larger loop (residues 43 through 54) constitutes a β -sheet flap (the "major flap"), while a smaller, "minor active-site loop" (residues 188 through 195) sits roughly opposite the major flap centered about the binuclear zinc site. Crystallographic temperature factors, the absence of electron density at the apex of the major flap (Concha et al. 1996), as well as reduced heteronuclear NOEs for residues that constitute the flap (Scrofani et al. 1999) are indications of flexibility within the active site region. Recently, we demonstrated through nuclear magnetic resonance (NMR) relaxation experiments that the polypeptide-chain dynamics of the major flap and minor active-site loop result in a plastic, flexible binding pocket (Huntley et al. 2000). Nanosecond timescale motions present for the flap and minor loop were completely damped out upon binding of a tight-binding inhibitor. We concluded that the observed plasticity of the lactamase binding pocket enables the enzyme to accommodate antibiotic substrates of different sizes and different chemical compositions. This plasticity is probably a factor in the broad specificity of this lactamase against the catalog of structurally diverse β -lactam antibiotic substrates presently available.

The flexibility of the tryptophan ring located at the apex of the major flap (Trp49) changes dramatically upon binding of an inhibitor, SB225666 (Fig. 2; Huntley et al. 2000). The $\{^1\text{H}\}\text{-}^{15}\text{N}$ heteronuclear NOE of the Trp49 indole NH indicates a high degree of flexibility of the tryptophan ring in the free form of the lactamase on a nano- to picosecond timescale. Upon binding of inhibitor, the heteronuclear NOE of the indole NH increased dramatically, indicating that the motions of the side chain have been damped out, so that its motions now approximate closely to those of the backbone and side chains of the folded protein. These results imply that the binding of inhibitor (and, by implication, substrate) likely involves an interaction with this tryptophan side chain, but its precise role in the recruitment and/or binding of substrates was not clear from these measurements. To better understand the role of the tryptophan side chain at the apex of the major flap, we have characterized a number of Trp49 mutants of the metallo- β -lactamase from *B. fragilis*. We have also prepared ^{15}N -labeled mutant proteins for study by NMR to ascertain the nature of any structural changes that may occur to the major flap, and to examine potential changes to the dynamics of the flap

Figure 1. Ribbon diagram for the metallo- β -lactamase from *Bacteroides fragilis* (Carfi et al. 1998). Zinc ions are shown as gold spheres. The indole side chain for Trp49 is shown in green. Magenta spheres represent the amide nitrogens of residues whose cross peaks in the ^1H - ^{15}N HSQC spectrum have been significantly affected by mutation of Trp49 to Lys or Phe.

Figure 2. Structures of the inhibitor SB225666 (A), ampicillin (B), and nitrocefin (C).

that may result from amino-acid substitution of the tryptophan. In this study, we describe the results of these investigations that more precisely characterize the role of Trp49 in the recognition and binding of antibiotic substrates.

Results

Steady-state kinetics of nitrocefin hydrolysis

Steady-state kinetic parameters for the wild type and mutant proteins were determined and are shown in Table 1. Inspection of the data reveals a wide range of k_{cat} and K_{m} values. All of the substitution mutant enzymes show an effect of the substitution of Trp49. The W49F shows K_{m} and k_{cat} values closest to those of the wild-type protein, indicating that the aromatic ring of Trp49 has an important contributory effect in both binding and catalysis. In general, smaller amino acids at position 49 show greater effects, a significant decrease in k_{cat} and increase in K_{m} . Consistent with this, the deletion mutant ($\Delta[47-49]$) shows the largest change in K_{m} and k_{cat} , to $430 \pm 90 \mu\text{M}$ and $120 \pm 10 \text{ s}^{-1}$ respectively.

NMR spectroscopy

The W49K mutant was characterized by NMR, to determine whether changes to the structure or dynamics of the major

flap had occurred as a result of the mutation and could be invoked to explain the difference between the kinetic parameters of this mutant and the wild-type enzyme. Figure 3 shows an overlay of representative 2D ^{15}N -HSQC spectra of the wild-type (red) and W49K (black) proteins, respectively. Excellent correspondence of wild-type and mutant ^{15}N and ^1H backbone amide chemical shifts is observed for a majority of the NMR resonances. However, amide backbone resonances for residues that flank the mutation site, residues 43 through 54 (excluding Pro53), did shift to variable degrees. Appreciable shifts were also observed for resonances Gly224 and Leu105 (located near the base of the major flap). In the W49F mutant spectrum, a significant shift was also seen for Asn 193 in the minor active site loop (data not shown); this cross peak was much less affected in the spectrum of the W49K mutant protein.

To determine if changes to the secondary structure of the major flap have occurred as a result of the mutation, 3D ^{15}N NOESY-NHSQC and 3D ^{15}N TOCSY NMR spectra were acquired for the ^{15}N -labeled W49K mutant protein at 800 MHz. Unambiguous identification of shifted backbone amides were made by observing a complete set of sequential interresidue $d_{\alpha\text{N}}(i,i+1)$ NOE connectivities in the β -hairpin. The β -hairpin secondary structure was confirmed by the observation of NOEs across the anti-parallel β -strands, between the amides of S42 and S54, A44 and V52, and I46 and V52. Although these resonances were in some cases significantly shifted in the mutant protein, a comparison of the NOEs with those reported by Scrofani et al. (1998) indicates that the integrity of the antiparallel β -sheet structure of the major flap is unaffected by the mutation.

The heteronuclear NOEs for residues of the wild-type protein, and for those for the ^{15}N -labeled W49K mutant are shown in Figure 4. The errors associated with the W49K data (black) are slightly larger relative to those of the wild-type enzyme (red). This is because two complete heteronuclear NOE data sets (saturated and unsaturated pairs) were obtained for the W49K mutant versus four for the wild-type enzyme. The backbone and Trp side chain NH heteronuclear NOEs for the wild-type and W49K proteins, including those of major flap, are almost identical, indicating that the dynamics of the flap are unchanged on the nanosecond to picosecond timescale in the mutant protein.

Table 1. Kinetic characterization of metallo- β -lactamase wild-type and mutant proteins

	k_{cat} (s^{-1})	K_{m} (μM)	$k_{\text{cat}}/K_{\text{m}}$ ($\mu\text{M}^{-1} \text{ s}^{-1}$)
WT ^a	226 ± 6	7.1 ± 0.7	32 ± 4
W49F	274 ± 28	13 ± 2	21 ± 5
W49A	283 ± 33	60 ± 13	4.7 ± 1.6
W49L	169 ± 10	127 ± 16	1.33 ± 0.25
W49K	163 ± 16	187 ± 24	0.87 ± 0.20
W49D	96 ± 15	171 ± 45	0.56 ± 0.23
$\Delta(47-49)$	120 ± 10	430 ± 90	0.28 ± 0.08

^a Values for wild-type lactamase from Wang et al. 1999

Discussion

Restriction of the Trp49 indole upon binding of substrates

Our previous investigations of this metallo- β -lactamase demonstrated that flexibility within the enzyme active site leads to a plastic binding pocket that can bind structurally diverse antibiotic substrates. These data, however, could not unambiguously characterize the role of the tryptophan indole located at the apex of the major flap. One X-ray crystal

Figure 3. Superposition of ^{15}N NMR spectrum of the wild-type metallo- β -lactamase (red) acquired at 750 MHz and of the W49K mutant enzyme (black) acquired at 600 MHz. Both samples contained 125 μM protein in 10 mM HEPES buffer (pH 7.0) containing 100 μM ZnCl_2 . Backbone amide resonances that shifted as a result of the mutation (generally those that flanked the mutation site) are indicated.

structure of the free lactamase, 1BMI, localized the indole extended away from the active site in a solvent-accessible position that seems unusual for such a bulky, hydrophobic residue. It has been suggested that the indole functions as a “fishing rod” to recruit substrates to the active site (Scrofani et al. 1999), or that it functions to bind substrates and/or cap the binding pocket in the presence of substrate (Fitzgerald et al. 1998; Concha et al. 2000). It is not certain what protein-substrate interactions may be responsible for such proposed functions of the indole. It is possible that the indole interacts favorably with specific features of the substrate (e.g., the thiazoline or phenyl moieties), or perhaps that it has a more general hydrophobic role in the binding of substrates. Nevertheless, the restriction of the indole side chain upon binding of SB225666, which is evident from the heteronuclear NOE data of the free and inhibitor-bound enzyme (Huntley et al. 2000), strongly suggests that the indole does indeed

have an important role in the binding of substrates, or stabilization of intermediates in the cleavage mechanism.

Comparison of previously determined inhibitor-bound lactamase structures

Previous crystal structures of the lactamase from *B. fragilis* complexed to 4-morpholine-ethanesulphonic acid (MES; Fitzgerald et al. 1998), or to the biphenyl tetrazole L-159,061 (Toney et al. 1998) localize the inhibitors within the enzyme binding pocket. As shown in Figure 5, it can be seen that the major flap bends slightly toward the enzyme-active site in the presence of the inhibitors (not shown for clarity). The tryptophan side chain is also observed to move down toward the active site to the same extent and to approximately the same position for each enzyme/inhibitor complex. In its interaction with MES, the indole abuts an

Figure 4. Plot of the ^1H - ^{15}N NOE measured at 600 MHz for the wild-type metallo- β -lactamase (Huntley et al. 2000; red) and of the W49K mutant enzyme (black) versus residue number. Heteronuclear NOE values for the tryptophan indole NHs (Trp1) are shown as a separate graph to the right of the figure. Heteronuclear NOE values were determined from the ratio of peak intensity in the absence and in the presence of proton saturation. For the determination of peak height uncertainties, four identical saturated/unsaturated experiments were acquired for the wild-type enzyme. Only two data sets were acquired for the W49K mutant.

aliphatic portion of the morpholino moiety (Fig. 6A). However, in its interaction with the biphenyl tetrazole (BPT), the tryptophan side chain roughly faces, and slightly overlaps, the biphenyl portion of the BPT (Fig. 6B; a more detailed description and analysis of these lactamase/inhibitor complexes is given in the references for Fitzgerald et al. 1998 and Toney et al. 1998).

The structure of the IMP-1 metallo- β -lactamase from *Pseudomonas aeruginosa* in the presence of a mercaptocarboxylate inhibitor was also able to localize the inhibitor

within the enzyme active site (Concha et al. 2000). While there are a number of differences between the two enzymes, like the lactamase from *B. fragilis*, the IMP-1 lactamase contains an extended major flap and has a tryptophan residue at its apex (Trp28). For the IMP-1/mercaptocarboxylate structure, the tryptophan side chain has been shown to interact “edge-to-face” with a thiophene portion of this particular inhibitor (Fig. 6C). These studies show quite clearly how the tryptophan interacts with each inhibitor individually; however, there is no consistent role that can be ascribed to the tryptophan in the binding of these specific substrates. Moreover, there is little correlation as to how these compounds are oriented, each taking up a distinctly different position within the enzyme active site.

Figure 5. Superposition of the free wild-type lactamase from *Bacteroides fragilis* (Carfi et al. 1998; gray), the MES-lactamase complex (Fitzgerald et al. 1998; blue), and the BPT-lactamase complex (Toney et al. 1998; gold). In the crystal structure of the free enzyme, the tryptophan is localized away from the active site in a solvent-accessible position. For the lactamase bound to 4-morpholine-ethanesulphonic acid and biphenyl tetrazole (ligands not shown for clarity) the major flap bends slightly toward the enzyme active site, with the indole side chain in approximately the same position for each lactamase/inhibitor complex.

The kinetic data support a nonspecific role for Trp49 in the binding of antibiotic substrates

The kinetic results for the deletion mutant ($\Delta 47$ –49) show that the tryptophan is critical for enzyme function as removal of the tryptophan weakens enzyme activity. This mutant is, in fact, no longer able to confer ampicillin resistance in vivo (W. Fast and S.J. Benkovic, unpubl.). However, the function of the tryptophan is not clear from the behavior of this mutant alone. We hypothesized that if there were a specific role of the tryptophan indole side chain in the binding and/or recruitment of substrates (e.g., recognition of a specific moiety of the lactam substrate), then the rather subtle change to a phenylalanine ought to result in pronounced differences in the enzyme kinetics of the wild type and W49F mutant proteins. Table 1 shows that the kinetics of these two enzymes in the nitrocefin hydrolysis reaction are quite similar. We therefore conclude that the effect of

Figure 6. Structures showing the positions of ligands in three complexes of metallo- β -lactamases (A) The 4-morpholine-ethanesulphonic acid complex with the enzyme from *Bacteroides fragilis* (1A7T; Fitzgerald et al. 1998). (B) The biphenyl tetrazole L-159,061 complex with the enzyme from *Bacteroides fragilis* (1A8T; Toney et al. 1998). (C) The mercapto-carboxylate complex with the enzyme from *Pseudomonas aeruginosa* (1DD6; Concha et al. 2000) inhibitors. The positions of the two zinc atoms in each complex are shown by blue balls, and the location of the Trp49 indole side chain in each structure is shown in green.

the Trp residue at position 49 is likely a nonspecific one, dependent on the aromatic or hydrophobic character of the side chain.

All of the other mutants show a significant difference from wild type in the kinetics of nitrocefin hydrolysis. Although all of the mutants are capable of reaction with nitrocefin, the changes result in a less efficient reaction. The effects of the different mutations can be summarized in

terms of three parameters. Firstly, a change in the size of the side chain (e.g., the W49A mutant) results in a decrease in affinity (increase in K_m) of ~ 1 order of magnitude, presumably as a result of the loss of hydrophobic surface area for interaction with the substrate. However, a second effect is seen in the behavior of the W49K and W49L mutants. Although the Lys and Leu side chains are larger than that of Ala, the K_m values for the nitrocefin hydrolysis reaction are further increased over that of the W49A mutant, by a factor of 2–3, suggesting that steric factors are also important in the substrate affinity. A third effect is indicated by a comparison of the K_m values of the pairs of mutants W49A/W49D and W49L/W49K. It appears that for side chains of approximately the same size and shape, the addition of a charged group causes a decrease in the affinity of the enzyme for nitrocefin. This observation can be rationalized if the Trp side chain of the wild-type enzyme provides a hydrophobic environment in the enzyme-substrate complex; such a structure would be destabilized by the presence of a charged group in the side chain.

The k_{cat} values of the W49K, -L, and $\Delta 47-49$ mutant proteins are significantly lower than for wild type. This may be because of the inability of the side chains (or lack thereof) to hold the substrate in its optimal orientation, or may be from other major flap interactions. Upon closing of the major flap, Trp49 is capable of interacting with Asn193 located within the minor active-site loop, a residue implicated in catalyzing the hydrolysis reaction (Yanchak et al. 2000). Previously, we have shown that binding of SB225666 resulted in the NMR shift of backbone amide resonances of Gly192 and Asn193 as well as the indole NH resonance of Trp49 (Scrofani et al. 1999). For the W49F mutant protein, the backbone amide resonance for Asn193 has shifted relative to the wild-type apoprotein. These findings bring up the possibility of interactions between the side chains of residues in the major flap and minor active-site loop, as has been suggested through molecular dynamics simulations of the wild-type enzyme in complex with SB225666 (Salsbury Jr. et al. 2001). Therefore, if mutations to Trp49 render the major flap incapable of contacting the minor active-site loop, this may represent the disruption of an induced-fit substrate binding mechanism present in the wild-type enzyme. More detailed kinetic and structural investigations will be necessary to probe these proposed major-flap/minor-loop interactions and their potential role in catalysis.

Because we do not observe changes in either the picosecond dynamics nor the secondary structure of the major flap as a result of amino-acid substitution at position 49 (Figs. 4, 5), we conclude that differences in the enzyme kinetics of the various mutant proteins relative to the wild-type enzyme are from the nature of the amino-acid substitution, rather than to an alteration of the structure or dynamics of the flap.

The tryptophan indole serves to cap the binding pocket in the presence of substrate

A comparison of the properties of the mutants at position 49 suggests that once the substrate is recruited to the active site, the tryptophan serves to cap the binding pocket, through either nonspecific hydrophobic or aromatic stacking interactions. Additional specific interactions may occur depending on the structure of the substrate. The importance of Trp 49 is demonstrated by the observation that, despite quite low-sequence identities, the metallo- β -lactamases from *P. aeruginosa* and *B. cereus* also contain a β -hairpin substrate-binding flap with tryptophan or phenylalanine (respectively) at the apex. These conclusions are consistent with the broad specificity of metallo- β -lactamases against a wide variety of structurally diverse antibiotic substrates. These findings identify the tryptophan as a site whose interactions should be included in the design of novel therapeutic agents or therapeutic strategies to combat organisms that harbor metallo- β -lactamases.

Materials and methods

Preparation of mutant proteins

In-vitro, site-directed mutagenesis was used to prepare W49F, -A, -L, -K, and -D mutant proteins. The plasmid pT7CcrANDEO2 (Yang et al. 1992) used for expression of the wild-type enzyme was employed as an extension template, while synthetic oligonucleotide primers (40 bases in length) containing the desired point mutation flanked by unmodified nucleotide sequence were obtained from Operon Technologies, Inc. Site-directed mutagenesis was accomplished using QuickChange Site-Directed Mutagenesis Kits (Stratagene). The presence of the desired mutation was confirmed by sequencing the complete lactamase gene from plasmid purified employing a MiniPrep purification kit (Qiagen). ^{15}N -labeled W49F, -A, -L, -K, and -D mutant metallo- β -lactamase proteins were expressed and purified according to previously described methods (Scrofani et al. 1999). Samples were suspended in 10 mM HEPES and 100 μM ZnCl_2 (pH 7.0) and concentrated to 125 μM using an Amicon Centriprep 10 ultrafilter.

An E47-G48-W49 deletion mutant ($\Delta[47-49]$) was prepared using the overlap extension method of Ho et al. (1989) using pMSZ01 (Crowder et al. 1996) as a template, the mutagenic primers 5'-CTC GCC GAA ATC * GGT ATG GTA CCT TCC-3', 5'-GGA AGG TAC CAT ACC * GAT TTC GGC GAC-3' (asterisks designate the sites where three codons were omitted from these primers to allow for the deletion), and the outside primers NcoI for and BamHI rev (Wang and Benkovic 1998). The final PCR product of this procedure was digested with NcoI and BamHI and ligated into an appropriately digested pET27b vector (Novagen) to form the expression plasmid pWFZ01. The sequence of this mutant was verified by sequencing the entire gene. The deletion mutant was expressed and purified as described (Wang and Benkovic 1998), except that this mutant exited the Q-sepharose column earlier than the wild-type protein because of the removal of a surface carboxylate (E47). This procedure resulted in a yield of ~4 mg of purified protein per liter of culture media.

Kinetic measurements

Steady-state kinetic studies of ^{15}N -labeled W49F, -A, -L, -K, and -D mutant proteins, as well as unlabeled $\Delta(47-49)$ mutant protein were performed by monitoring the enzyme-catalyzed hydrolysis of nitrocefin at 25°C (pH 7.0). An extinction coefficient of 35,600 $\text{M}^{-1} \text{cm}^{-1}$ was used to determine the concentration of the mutant protein samples (calculated using the program SEDNTERP [http://www.bbri.org/RASMB/rasmb.html]). The rate of nitrocefin hydrolysis was determined by monitoring absorbance at 485 nm ($\Delta\epsilon = 15,900 \text{ M}^{-1} \text{cm}^{-1}$) for product formation from nanomolar (0–4 nM) concentrations of enzyme and 0–2500 μM nitrocefin in pH 7.0 buffer free of heavy metal contamination. For nitrocefin concentrations >1000 μM , a 2 mm path-length cuvette was used.

NMR spectroscopy

NMR spectra were acquired at 295 K on Bruker DRX-600, DMX-750, or DRX-800 spectrometers equipped with either 5-mm, inverse-detected, triple-resonance, single-axis gradient probe (Nalorac) or with 5-mm, inverse-detected, triple-resonance, triple-axis gradient probes (Bruker). ^{15}N HSQC experiments were performed with ^{15}N -labeled W49F, -A, -L, -K, and -D mutant proteins containing 5% D_2O . Spectra were processed using NMRPipe/NMRDraw (Delaglio et al. 1995) and analyzed with NMRView (Johnson and Blevins 1994). Data sets were zero-filled and apodized with exponential, cosine-squared, or Lorentzian-to-Gaussian window functions. Linear prediction was used to extend the ^{15}N indirect dimension thereby improving digital resolution. NMR probe temperature was carefully calibrated using neat methanol (Van Geet 1970).

Heteronuclear NOE spectra were recorded at 600 MHz for the W49K mutant protein as 128 X 1024 complex data points with 32 transients/point. Spectral widths were 1580 and 9615 Hz along the ^{15}N and ^1H dimensions, respectively. Heteronuclear NOE values were determined from the ratio of peak intensity in the absence and presence of proton saturation (Nicholson et al. 1992). Saturation was achieved by use of a train of 120° pulses separated by 5 ms for a total irradiation time of 3 s (Farrow et al. 1995). A 4-s recycle delay was employed for all heteronuclear NOE experiments. For the determination of peak height uncertainties, two identical saturated/unsaturated experiments were acquired. Analysis of backbone amide heteronuclear NOEs for the W49K mutant used backbone amide and tryptophan indole NH resonance assignments previously determined for the wild-type lactamase (Scrofani et al. 1998). In the case where significant chemical shift was observed for backbone amides (generally those that flank the mutation site), resonance assignment was accomplished by use of 3D ^{15}N NOESY-HSQC and 3D ^{15}N TOCSY spectra acquired at 800 MHz on uniformly ^{15}N -labeled W49K protein. Chemical shifts were referenced to the water resonance at 4.76 ppm.

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References

- Bush, K. 1989a. Classification of the β -lactamases: Groups 1, 2a, 2b and 2b'. *Antimicrob. Agents Chemother.* **33**: 264–271.
- . 1989b. Classification of β -lactamases: Groups 2c, 2d, 2e, 3 and 4. *Antimicrob. Agents Chemother.* **33**: 271–276.
- Bush, K., Jacoby, G.A., and Medeiros, A.A. 1995. A functional classification scheme for β -lactamases and its correlation with molecular structure. *Antimicrob. Agents Chemother.* **39**: 1211–1233.
- Carfi, A., Duée, E., Paul-Soto, R., Galleni, M., Frère, J.-M., and Dideberg, O. 1998. X-ray structure of the ZnII β -lactamase from *Bacteroides fragilis* in an orthorhombic crystal form. *Acta Crystallogr. D Biol. Crystallogr.* **54**: 47–57.
- Concha, N.O., Rasmussen, B.A., Bush, K., and Herzberg, O. 1996. Crystal structure of the wide-spectrum β -lactamase from *Bacteroides fragilis*. *Structure* **4**: 823–836.
- Concha, N.O., Janson, C.A., Rowling, P., Pearson, S., Cheever, C.A., Clarke, B.P., Lewis, C., Galleni, M., Frère, J.M., Payne, D.J., et al. 2000. Crystal structure of the IMP-1 metallo β -lactamase from *Pseudomonas aeruginosa* and its complex with a mercaptocarboxylate inhibitor: Binding determinants of a potent, broad-spectrum inhibitor. *Biochemistry* **39**: 4288–4298.
- Crowder, M.W., Wang, Z., Franklin, S., Zovinka, E., Que Jr., L., and Benkovic, S.J. 1996. Characterization of the metal-binding sites of β -lactamase from *Bacteroides fragilis*. *Biochemistry* **35**: 12126–12132.
- Cuchural, G.J., Malamy, M.H., and Tally, F.P. 1986. β -lactamase-mediated imipenem resistance in *Bacteroides fragilis*. *Antimicrob. Agents Chemother.* **30**: 645–648.
- Delaglio, F., Grzesiek, S., Vuister, G.W., Guang, Z., Pfeifer, J., and Bax, A. 1995. NMRPipe: A multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR* **6**: 277–293.
- Farrow, N.A., Zhang, O., Forman-Kay, J.D., and Kay, L.E. 1995. Comparison of the backbone dynamics of a folded and an unfolded SH3 domain existing in solution in aqueous buffer. *Biochemistry* **34**: 868–878.
- Fitzgerald, P.M., Wu, J.K., and Toney, J.H. 1998. Unanticipated inhibition of the metallo- β -lactamase from *Bacteroides fragilis* by 4-morpholineethanesulfonic acid (MES): A crystallographic study at 1.85 Å resolution. *Biochemistry* **37**: 6791–6800.
- Galleni, M., Lamotte-Brasseur, J., Rossolini, G.M., Spencer, J., Dideberg, O., and Frère, J.M. 2001. Standard numbering scheme for class B β -lactamases. *Antimicrob. Agents Chemother.* **45**: 660–663.
- Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K., and Pease, L.R. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77**: 51–59.
- Hughes, J.M. and Tenover, F.C. 1997. Approaches to limiting emergence of antimicrobial resistance in bacteria in human populations. *Clin. Infect. Dis.* **24**: S131–S135.
- Huntley, J.J.A., Scrofani, S.D.B., Osborne, M.J., Wright, P.E., and Dyson, H.J. 2000. Dynamics of the metallo- β -lactamase from *Bacteroides fragilis* in the presence and absence of a tight-binding inhibitor. *Biochemistry* **39**: 13356–13364.
- Johnson, B.A. and Blevins, R.A. 1994. NMRView: A computer program for the visualization and analysis of NMR data. *J. Chem. Phys.* **29**: 1012–1014.
- Livermore, D.M. 1998. β -lactamase-mediated resistance and opportunities for its control. *J. Antimicrob. Chemother.* **41 Suppl D**: 25–41.
- Matagne, A., Dubus, A., Galleni, M., and Frère, J.M. 1999. The β -lactamase cycle: A tale of selective pressure and bacterial ingenuity. *Nat. Prod. Rep.* **16**: 1–19.
- Nicholson, L.K., Kay, L.E., Baldisseri, D.M., Arango, J., Young, P.E., Bax, A., and Torchia, D.A. 1992. Dynamics of methyl groups in proteins as studied by proton-detected ^{13}C NMR spectroscopy. Application to the leucine residues of staphylococcal nuclease. *Biochemistry* **31**: 5253–5263.
- Normark, B.H. and Normark, S. 2002. Evolution and spread of antibiotic resistance. *J. Intern. Med.* **252**: 91–106.
- Sabath, L.D. and Abraham, E.P. 1966. Zinc as a cofactor for cephalosporinase from *Bacillus cereus* 569. *Biochem. J.* **98**: 11c–13c.
- Salsbury Jr., F.R., Crowley, M.F., and Brooks III, C.L. 2001. Modeling of the metallo- β -lactamase from *B. fragilis*: Structural and dynamic effects of inhibitor binding. *Proteins* **44**: 448–459.
- Sandanayaka, V.P. and Prasad, A.S. 2002. Resistance to β -lactam antibiotics: Structure and mechanism based design of β -lactamase inhibitors. *Curr. Med. Chem.* **9**: 1145–1165.
- Scrofani, S.D., Wright, P.E., and Dyson, H.J. 1998. ^1H , ^{13}C and ^{15}N NMR backbone assignments of 25.5 kDa metallo- β -lactamase from *Bacteroides fragilis*. *J. Biomol. NMR* **12**: 201–202.
- Scrofani, S.D.B., Chung, J., Huntley, J.J.A., Benkovic, S.J., Wright, P.E., and Dyson, H.J. 1999. NMR characterization of the metallo- β -lactamase from *Bacteroides fragilis* and its interaction with a tight-binding inhibitor: Role of an active-site loop. *Biochemistry* **38**: 14507–14514.
- Senda, K., Arakawa, Y., Ichiyama, S., Nakashima, K., Ito, H., Ohsuka, S., Shimokata, K., Kato, N., and Ohta, M. 1996. PCR detection of metallo- β -lactamase gene (*bla_{IMP}*) in Gram-negative rods resistant to broad-spectrum β -lactams. *J. Clin. Microbiol.* **34**: 2909–2913.
- Spencer, J., Clarke, A.R., and Walsh, T.R. 2001. Novel mechanism of hydrolysis of therapeutic β -lactams by *Stenotrophomonas maltophilia* L1 metallo- β -lactamase. *J. Biol. Chem.* **276**: 33638–33644.
- Toney, J.H., Fitzgerald, P.M.D., Grover-Sharma, N., Olson, S.H., May, W.J., Sundelof, J.G., Vanderwall, D.E., Cleary, K.A., Grant, S.K., Wu, J.K., et al. 1998. Antibiotic sensitization using biphenyl tetrazoles as potent inhibitors of *Bacteroides fragilis* metallo- β -lactamase. *Chem. Biol.* **5**: 185–198.
- Ullah, J.H., Walsh, T.R., Taylor, I.A., Emery, D.C., Verma, C.S., Gamblin, S.J., and Spencer, J. 1998. The crystal structure of the L1 metallo- β -lactamase from *Stenotrophomonas maltophilia* at 1.7 Å resolution. *J. Mol. Biol.* **284**: 125–136.
- Van Geet, A.L. 1970. Calibration of methanol nuclear magnetic resonance thermometer at low temperature. *Analyt. Chem.* **42**: 679–680.
- Wang, Z.G. and Benkovic, S.J. 1998. Purification, characterization, and kinetic studies of a soluble *Bacteroides fragilis* metallo- β -lactamase that provides multiple antibiotic resistance. *J. Biol. Chem.* **273**: 22402–22408.
- Wang, Z.G., Fast, W., and Benkovic, S.J. 1998. Direct observation of an enzyme-bound intermediate in the catalytic cycle of the metallo- β -lactamase from *Bacteroides fragilis*. *J. Am. Chem. Soc.* **120**: 10788–10789.
- . 1999. On the mechanism of the metallo- β -lactamase from *Bacteroides fragilis*. *Biochemistry* **38**: 10013–10023.
- Yanchak, M.P., Taylor, R.A., and Crowder, M.W. 2000. Mutational analysis of metallo- β -lactamase CcrA from *Bacteroides fragilis*. *Biochemistry* **39**: 11330–11339.
- Yang, Y., Wu, P., and Livermore, D.M. 1990. Biochemical characterization of a β -lactamase that hydrolyzes penems and carbapenems from two *Serratia marcescens* isolates. *Antimicrob. Agents Chemother.* **34**: 755–758.
- Yang, Y., Rasmussen, B.A., and Bush, K. 1992. Biochemical characterization of the metallo- β -lactamase CcrA from *Bacteroides fragilis* TAL3636. *Antimicrob. Agents Chemother.* **36**: 1155–1157.