Characterization of the Metal-Binding Sites of the β-Lactamase from *Bacteroides fragilis*†

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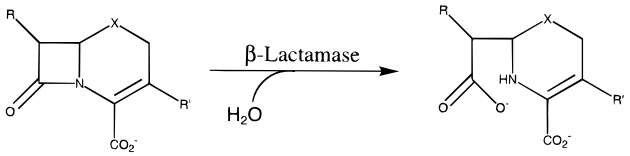
ABSTRACT: In an effort to better understand the structure and function of the metallo-β-lactamase from *Bacteroides fragilis*, spectroscopic and metal-binding studies were performed on the native, metal-substituted, and mutant forms of the enzyme. Atomic absorption studies demonstrate that the native *B. fragilis* enzyme tightly binds 2 mol of Zn(II) and, along with mutagenesis studies, that the presence of both metal ions is required for full catalytic activity. EPR spectroscopy was used to confirm that the Co(II)-substituted β-lactamase binds 2 mol of Co(II) per mole of enzyme, that the two Co(II)'s are high-spin and probably uncoupled, with apparent *g* values of 6.5, 4.2, and 2.0, and that the coordination number of the Co(II) is 5 or 6. This number of ligands for the Co(II)-substituted enzyme is confirmed by UV−Vis spectra, which demonstrate the presence of very weak d−d transitions between 550 and 650 nm ($\epsilon \approx 30$ $M^{-1}\cdot cm^{-1}$) and an intense feature at 320 nm ($\epsilon \approx 1570$ $M^{-1}\cdot cm^{-1}$). The latter is assigned to a cysteine sulfur to Co(II) ligand-to-metal charge transfer band, and this assignment is confirmed by the disappearance of this band in the UV−Vis spectrum of a Co(II)-substituted C168S mutant. ^{1}H NMR studies on the Co(II)-substituted enzyme suggest the presence of three histidine ligands bound to Co(II). Taken together, these studies support the sequence comparison study of Rasmussen et al., in which there is a catalytic metal-binding site with three histidines and one cysteine (C168). The remaining ligands are postulated to be water molecules involved in catalysis. Mutagenesis studies, in combination with activity assays and metal-binding studies, have been used to identify Asp61, Asp90, Asp152, and Asp183 as possible ligands to the second metal-binding site, with Asp90 and Asp152 having a pronounced effect on k_{cat}. These results are discussed in light of the recent crystal structure of the metallo-β-lactamase from *B. cereus*.

Strains of antibiotic-resistant bacteria have arisen owing to their possession of β-lactamases that catalyze the hydrolysis of the fused four-membered ring of β-lactams found in penicillin and cephalosporin drugs (Scheme 1). The resulting monocyclic product no longer inhibits a transpeptidase that is necessary for bacterial cell wall synthesis (Knowles, 1985). Currently, β-lactamases are classified in four groups (Bush, 1989a,b; Bush et al., 1995): members of groups 1, 2, and 4 utilize an active-site serine for nucleophilic attack on the β-lactam carbonyl (Knowles, 1985), while members of group 3 enzymes require metal ions for activity, are inhibited by ethylenediaminetetraacetic acid (EDTA),[1] hydrolyze all tested penicillin- and cephalosporin-type antibiotics, and are unaffected by clavulanic acid, a classical β-lactamase inactivator (Bush, 1989b). Differences in the interaction of the enzymes with substrates and/or inhibitors provide the basis for subgrouping the serine-β-lactamases. Within group 3, there are at least 10 bacterial sources of the enzyme: *Bacillus cereus* (Sabath & Abraham, 1966), *Xanthomonas maltophilia* (Saino et al., 1982), *Bacillus licheniformis* (Mezes et al., 1983), *Flavobacterium odoratum* (Sato et al., 1985), *Legionella gormaii* (Fujii et al., 1986), *Pseudomonas aeruginosa* (Livermore & Jones, 1986), *Bacteroides fragilis* (Cuchural et al., 1986), *Aeromonas hydrophilia* (Iaconis & Sanders, 1990), *Serratia marcescens* (Yang et al., 1990), and *Pseudomonas cepacia* (Baxter & Lambert, 1994). Several of these organisms are infectious to humans; in fact, some *X. maltophilia* strains have been linked to a wide outbreak of nosocomial infections in patients with weakened immune systems (Khardori et al., 1990). At present, no clinically-useful inhibitor of the group 3 β-lactamases is known; therefore, the detailed structural and mechanistic characterization of these enzymes is an important first step for the rational design of a therapeutic inhibitor.

Scheme 1



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[1] Abbreviations: ϵ, extinction coefficient; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; G, gauss; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Im, imidazole; K_D, dissociation constant; *k*, Boltzmann constant; LMCT, ligand to metal charge transfer; MCD, magnetic circular dichroism; NMR, nuclear magnetic resonance; o.d., outer diameter; *P*, power; $P_{1/2}$, power at half-saturation; PCR, polymerase chain reaction; *S*, signal; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; UV−Vis, ultraviolet−visible.

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The most thoroughly characterized enzymes in group 3 β-lactamases are from *Bacillus cereus*, although the *B. fragilis* and *Aeromonas hydrophilia* enzymes have been studied using steady-state kinetics (Yang et al., 1992; Felici et al., 1993). A low-resolution crystal structure of the *B. cereus* enzyme revealed 1 mol of Zn(II) in the active site bound by three histidines (His86, His88, and His210) and one cysteine (Cys168) (Sutton et al., 1987). A second metal-binding site that utilizes two aspartic acids as ligands (Asp81 and Asp90) was also identified. The two sites differ markedly in their affinity for the metal ion; for the first, the K_D is *ca.* 1 μM, while for the second, the K_D is *ca.* 24 mM (Davies & Abraham, 1974; Baldwin et al., 1978). Using sequence comparisons, Rasmussen et al. (1990) and Walsh et al. (1994) subsequently reported that the metal-binding ligands in the catalytic site of the *B. cereus* enzyme were strictly conserved in the *B. fragilis* and *X. maltophilia* enzymes with the exception in the *X. maltophilia* enzyme where Cys168 is replaced by a Ser. One of the two ligands (Asp90) in the second metal-binding site of the *B. cereus* enzyme is conserved in enzymes from the other two sources.

Recently, a second crystal structure of the *B. cereus* enzyme has been determined to 2.5 Å (Carfi et al., 1995). This structure differs from the earlier one; in particular, Cys168 is not a ligand to Zn(II), and His149 rather than His210 is a ligand. Moreover, only a single metal-binding site is present. The discrepancies between the two crystallographic studies were not addressed (Carfi et al., 1995).

The rational design and preparation of a therapeutically useful inhibitor require a detailed understanding of the metallo-active site. We report here, that in contrast to the *B. cereus* enzyme, the enzyme from *B. fragilis* **tightly** binds 2 mol of metal ions per mole of enzyme. Results from spectroscopic and mutagenesis studies confirm that one cysteine and three histidines do in fact serve as metal-binding ligands; one metal-binding site is probably identical to the catalytic site of the *B. cereus* enzyme as initially described. Further investigation based on mutagenesis and metal-binding studies has been used to identify Asp61, Asp90, Asp152, and Asp183 as possible metal-binding ligands in the second site and Asp56 as a potentially important catalytic residue. Finally, our results are contrasted to the recent crystal structure of *B. cereus* (Carfi et al., 1995).

EXPERIMENTAL PROCEDURES

Enzyme Preparation. The β-lactamase from *B. fragilis* (Ccr) was produced from an overexpressing *E. coli* BL21(DE3) clone kindly provided by Dr. Beth Rasmussen at American Cyanamid Co. The protein was isolated as described previously (Yang et al., 1992) except that the cell culture was made 0.01% in glucose to suppress catabolites and grown at 30 °C to control uninduced protein production and to reduce cell lysis during growth. The obtained inclusion bodies were washed in 10 mM HEPES, pH 7.4, and dissolved in 8 M urea, 10 mM HEPES, 1 M NaCl, pH 7.4. The protein was then refolded by dialyzing against 10 mM HEPES, 1 M NaCl, pH 7.4, at room temperature to yield more pure and active protein. The metal-substituted or apo forms of the enzyme were prepared either by inclusion of 10−200 μM of the desired metal ion in the the refolding buffer or by deletion of any metal ion from all the buffers, respectively. The purity of the resulting enzyme was ascertained by SDS−PAGE, and a single protein band at *ca.* 26 kDa was obtained in all cases. The protein was quantitated using $\epsilon_{280nm} = 2.08$ mL/(mg·cm), and the activity assays were conducted in 50 mM phosphate buffer, pH 7.0, using nitrocefin (obtained from SmithKline Beecham) as substrate; the formation of hydrolyzed nitrocefin was monitored and quantitated using $\epsilon_{485nm} = 17\,400$ $M^{-1}\cdot cm^{-1}$ (O'Callaghan et al., 1972).

Table 1: List of Mutagenic Primers (5′ → 3′)

primer	sequence
pUCMSZFor	CTATGCGGCATCAGAGCAGATT
M13Rev	GATAACAATTTCACACAGGA
D56VFor	GCAGCGTTGCTG**GTC**ACACCGATCAAT
D56VRev	ATTGATCGGTCT**GAC**CAGCAACGCTGC
D61VFor	ACACCGATCAAT**GTC**GCACAAACGGAA
D61VRev	TTCCGTTTGTGC**GAC**ATTGATCGGTGT
D90VFor	CACTGGCACGGC**GTT**TGTATTGGCGGA
D90VRev	TCCGCCAATACA**AAC**GCCGTGCCAGTG
D152VFor	GGACATGCGACC**GTC**AATATCGTGGTT
D152VRev	AACCACGATATT**GAC**GGTCGCATGTCC
C168SFor	CCTTTTTGGCGGA**TCT**ATGCTTAAAGACA
C168SRev	TGTCTTTAAGCAT**AGA**TCCGCCAAAAAGG
D183VFor	AACATCTCG**GTC**GCGGACGTGACGGCA
D183VRev	TGCCGTCACGTCCGC**GAC**CGAGATGTT

Site-Directed Mutagenesis. The expression plasmid pT7CcrANDEO2 (Yang et al., 1992) was digested with *Nde*I and *Bam*HI, and the resulting 1.8 kb β-lactamase gene-containing DNA fragment was gel-purified and ligated into pUC19 to form the cloning vector. Site-directed mutants were then constructed using the modified overlap and extension method (Ho et al., 1990). In brief, in the first round of PCR, two DNA fragments (forward and reverse) containing the desired sequences were separately amplified, each using one mutagenic primer (forward or reverse) and a universal reverse (M13Rev) or forward (pUCMSZFor) primer. The resulting fragments were gel-purified and subjected to a second round of PCR. The entire β-lactamase gene was finally amplified using the above DNA fragments as template and M13Rev and pUCMSZFor as primers, digested with *Nde*I and *Bam*HI, and ligated into pUC19 to give a new plasmid containing the desired mutation. The mutation was verified by sequencing the entire gene, and the mutant β-lactamase gene was finally subcloned back into the original expression vector. The primers used in this work are shown in Table 1.

Metal Content Measurements. The metal content of the native, the metal-substituted, the mutant, and the apo β-lactamases was ascertained using either a Perkin Elmer 730 atomic absorption spectrophotometer in the flame mode or a Perkin Elmer 1100B atomic absorption spectrophotometer in the graphite furnace mode. At least three standard concentrations were used for the calibration curve, and each sample was read in triplicate. All protein samples were subjected to 4 days of dialysis against metal-free 10 mM HEPES, pH 7.4, buffer at 4 °C with multiple changes of dialysis buffers. The metal content values reported for enzyme samples are an average of readings from several preparations of the enzyme and corrected for the metal content of the final dialysis buffer.

UV−Vis Spectroscopy. UV−Vis spectra were collected on a Cary 1 UV−Vis spectrophotometer at 25 °C. Difference spectra were obtained by subtracting the UV−Vis

spectrum of the Zn(II)-containing β-lactamase from that of the Co(II)-substituted enzyme, after normalizing the spectra for protein concentration. The UV−Vis spectra of Co(II)-substituted carbonic anhydrase, prepared as described by Lindskog et al. (1968), and of Co(II)-substituted wild-type and C168S mutant β-lactamases were also buffer-subtracted. The buffer used in these spectra was 10 mM HEPES, pH 7.4.

Electron Paramagnetic Resonance Spectroscopy. EPR spectra were collected on a Bruker ESR 300E spectrometer equipped with an Oxford ESR 900 continuous-flow cryostat and an Oxford Model ITC4 temperature controller. Operating temperatures were read directly from the controller, which was calibrated with a carbon glass sensor. The buffer-subtracted EPR spectra were quantitated by double-integration of signal-averaged scans using a 200 μM $CoCl_2$ standard in 10 mM HEPES, pH 7.4, buffer.

The energy difference between the ground state and first excited state levels was estimated by the temperature dependence of the signal. Power saturation studies at different temperatures yielded $P_{1/2}$ values which were plotted using the equation $\ln P_{1/2} = -\Delta/kT + \ln A$ (Pilbrow, 1990; Yim et al., 1982). The value Δ is defined as the zero-field splitting energy, and A is a coefficient characteristic of the spin system.

Nuclear Magnetic Resonance Spectroscopy. ^{1}H NMR spectra were acquired on a Bruker AMX-500 spectrometer at 500 MHz at room temperature. A modified-DEFT pulse sequence (D1−90°−τ−180°−τ−90°−AQ) was employed to suppress water and other resonances due to the protein backbone in the diamagnetic region (Hochman & Kellerhals, 1980). The NMR chemical shift values are reported relative to the H_2O or HOD resonance at 4.8 ppm, with positive values indicating downfield shifts.

NMR samples were made by lyophilizing freshly-prepared Co(II)-substituted β-lactamase, followed by dissolving the freeze-dried sample in 50 mM phosphate buffer, pH 7.0, containing 10% D_2O, to give a protein concentration of ~1 mM. The D_2O sample was prepared similarly except that the freeze-dried sample was dissolved in 50 mM phosphate, pD 7.4. The Co(II) complexes, $Co(Me\text{-}Im)_2Cl_2$, $Co(Im)_2Cl_2$ and $Co(4\text{-}Me\text{-}Im)_2Cl_2$, were synthesized using previously published procedures (Davis & Smith, 1971; Goodgame et al., 1969a,b; Dash & Pujari, 1977) and used to determine the chemical shift range for the N−H protons on imidazoles bound to Co(II).

RESULTS AND DISCUSSION

Metal Content. Evidence from early crystallographic and spectroscopic studies indicates that group 3 β-lactamase from *B. cereus* contains two metal-binding sites with vastly different metal-binding affinities (Sutton et al., 1987; Baldwin et al., 1978). The tighter binding metal ion was postulated to be within the catalytic site with no role for the second metal ion offered. Given the close proximity of these two metal ion-binding sites, both metal ions may be involved in the catalytic cycle of the *B. cereus* enzyme. In contrast, a recent X-ray diffraction study of Carfi et al. (1995) identified only one Zn(II)-binding site in the *B. cereus* enzyme. All ligands comprising the high-affinity metal-binding site in the *B. cereus* enzyme are strictly conserved in the *B. fragilis* enzyme (Rasmussen et al., 1990), but only one of two ligands in the weaker metal ion-binding site is retained. Given the sequence homology between the *B. cereus* and *B. fragilis* enzymes, we felt that it was important to focus on the issue of whether one or two metal-binding sites is a common and possibly necessary characteristic of group 3 β-lactamases.

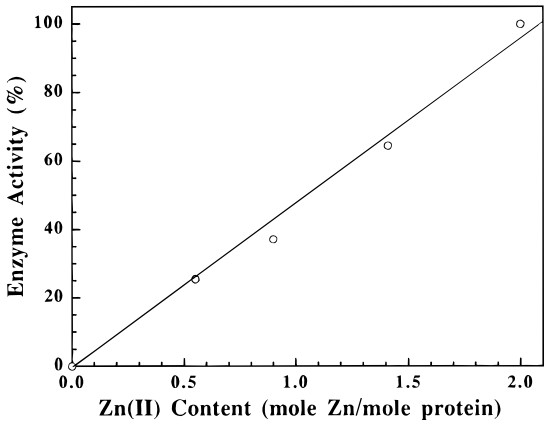

FIGURE 1: Correlation between the k_{cat} value and Zn(II) content of *B. fragilis* metallo-β-lactamase. Enzymes were purified with different refolding buffers containing 0, 5, 10, 50, and 100 μM Zn(II). The k_{cat} and Zn(II) content measurements were carried out according to Experimental Procedures.

The Zn(II)-containing β-lactamase from *B. fragilis* was prepared by including Zn(II) in the refolding buffer at concentrations as high as 200 μM, followed by dialysis of the refolded protein at 4 °C for 4 days against several changes of metal-free 10 mM HEPES, pH 7.4. Flame mode atomic absorption measurements were then carried out to determine the Zn(II) content of the protein samples, which depended on the Zn(II) concentration in the refolding buffer. The maximum metal content reached two Zn(II) per protein molecule when greater than 100 μM Zn(II) was included in the refolding buffer. The level of bound Zn(II) correlates linearly with the k_{cat} value of the enzyme (Figure 1 and Table 3), suggesting that both Zn(II) ions participate in catalysis and are required for maximizing the enzymatic activity. No other metal ion was detected in significant amounts within the enzyme (Table 2). When metal ions were excluded from the refolding media, the enzyme contained no appreciable amount of any metal ion and was inactive in the hydrolysis of nitrocefin (Figure 1 and Table 2).

The K_D values for metal ion binding were not obtained by fluorescence titration of the apoenzyme since the protein, after refolding from inclusion bodies, does not appear to bind metal ions reversibly (Stewart et al., 1994; Crowder et al., 1995). The addition of metal ion to the apo-protein does not yield an active enzyme. Since the two metal ion-binding sites can be saturated by 100 μM Zn(II) in the refolding buffer, the K_D of *both* metal-binding sites has an upper limit of $\leq$10 μM for Zn(II). The fact that the metal ion to protein stoichiometry remains at 2:1 after successive dialyses versus metal-free buffer suggests that the actual K_D's are much lower than 10 μM. This result reveals an important difference between the β-lactamases from *B. fragilis* and *B. cereus*, where the metal ion binding at the second site is very weak. Presently, there is no available information on metal stoichiometries of the other group 3 β-lactamases.

Electron Paramagnetic Resonance Spectroscopy. The substitution of Co(II) for Zn(II) in Zn(II)-containing proteins has been a very successful way to characterize Zn(II)-binding

Table 2: Metal to Enzyme Stoichiometries of *B. fragilis* β-Lactamase

sample	Zn(II)	Co(II)	Cu(II)	Fe(II)/(III)	Ni(II)	Mn(II)
Zn(II)-enzyme	2.0 ± 0.3	<0.005	<0.005	0.045	0.005	<0.005
Co(II)-enzyme	<0.005	2.1 ± 0.4	0.009	<0.005	<0.005	<0.005
apoenzyme	<0.005	<0.005	0.009	<0.005	<0.005	<0.005

Table 3: Metal Content and Kinetic Parameters for *B. fragilis* β-Lactamase Mutants

mutant	metal content	K_M (μM)	k_{cat} (s^{-1})
Zn(II)-wild type	2.0 ± 0.3	5.9 ± 1.4	154.3 ± 2.8
Co(II)-wild type	2.1 ± 0.4	15.2 ± 2.6	46.3 ± 2.3
Zn(II)-C168S	1.9 ± 0.3	30.4 ± 4.7	0.014 ± 0.001
Co(II)-C168S	1.9 ± 0.2	65.8 ± 17.0	0.19 ± 0.02
Zn(II)-D56V	2.1 ± 0.2	4.2 ± 0.5	0.15 ± 0.01
Zn(II)-D61V	0.93 ± 0.10	5.5 ± 0.4	55.5 ± 3.3
Zn(II)-D90V	0.43 ± 0.09	31.0 ± 2.0	0.024 ± 0.06
Zn(II)-D152V	0.67 ± 0.11	4.8 ± 0.4	0.019 ± 0.001
Zn(II)-D183V	0.54 ± 0.08	20.8 ± 3.1	33.9 ± 1.5

sites structurally (Bertini et al., 1982; Horrocks et al., 1980; Kiefer et al., 1993). By including Co(II) ($\geq$100 μM) in the refolding buffer during the β-lactamase purification, a Co(II)-substituted enzyme containing two Co(II) ions per protein molecule was obtained (Table 2). Since the Co(II)-substituted enzyme was catalytically active with a k_{cat} *ca.* 30% of that of the Zn(II)-containing protein (Table 3), the spectral properties of the Co(II)-substituted enzyme might offer valuable information about the metal-binding environment.

The low-temperature EPR spectrum of the Co(II)-substituted β-lactamase is shown in Figure 2 and is characterized by a broad signal with apparent *g* values of 6.5, 4.2, and 2.0. Using $CoCl_2$ as a standard, the EPR signal is integrated to 1.9 mol of Co(II) per mole of enzyme, which is in excellent agreement with the atomic absorption measurements (Table 2). The spectrum is also very similar in shape and *g* values to that of the Co(II)-substituted β-lactamase from *B. cereus* (Bicknell et al., 1986), suggesting that the two Co(II) ions in the *B. fragilis* enzyme are high-spin and not coupled. The fact that the signal is temperature-dependent and disappears at temperatures greater than 30 K (M. W. Crowder, unpublished results) supports the presence of two distinct, high-spin Co(II)-binding sites. Experiments to evaluate the metal ion−metal ion interatomic distance are currently underway.

The quantitation of the temperature-dependent EPR spectra of the Co(II)-substituted enzyme also provided information on the metal coordination. Analysis of the power saturation properties at different temperatures, i.e., $S/\sqrt{P}$ vs log *P* plots (Yim et al., 1982), furnished graphs of ln $P_{1/2}$ vs $1/T$ leading to the evaluation of the zero-field splitting energy (Δ). Values of Δ have been correlated with coordination numbers of high-spin Co(II) complexes: $\Delta = <15$ cm^{-1} (four-coordinate); $15 \leq \Delta \leq 50$ cm^{-1} (five-coordinate), and $\Delta \geq 50$ cm^{-1} (six-coordinate) (Pilbrow, 1990). The Δ value calculated for the Co(II)-substituted *B. fragilis* enzyme was 44 ± 2 cm^{-1} (Figure 3), suggesting that one or both Co(II)'s are five/six-coordinated.

UV−Vis Spectroscopy. The UV−Vis difference spectrum [spectrum of Co(II)-substituted wild-type enzyme minus the spectrum of the Zn(II)-containing wild-type enzyme] is characterized by an intense absorption at 320 nm ($\epsilon \approx 1570$ $M^{-1}\cdot cm^{-1}$) and several broad weak features between 550 and 650 nm ($\epsilon \approx 30$ $M^{-1}\cdot cm^{-1}$) (Figure 4, solid line). The former feature is characteristic of one S-to-Co(II) ligand to metal charge transfer (LMCT) transition (Alexander et al., 1993), implicating a cysteine as a metal-binding ligand in one of the sites. This result favors the pattern of metal ligands observed in the initial X-ray crystal structure for the *B. cereus* enzyme (Sutton et al., 1987), but not in the later structure solved by Carfi et al. (1995). It is possible that the slightly larger radius of Co(II) [as compared to Zn(II)] and the symbiotic nature of the Co(II)−S ligation (soft acid−soft base) may have resulted in a chance Cys−Co(II) linkage that is not present in the Zn(II) protein. However, this scenario is unprecedented in the literature and probably not a valid explanation for the discrepancy.

To confirm that Cys168, which is conserved in four of the five metallo-β-lactamase sequences (Rasmussen et al., 1990; Carfi et al., 1995), is a metal-binding ligand in the *B. fragilis* enzyme, site-directed mutagenesis on Cys168 was carried out. The UV−Vis difference spectrum of the Co(II)-substituted C168S mutant demonstrates that the S-to-Co(II) LMCT, observed in the wild-type Co(II)-substituted enzyme,

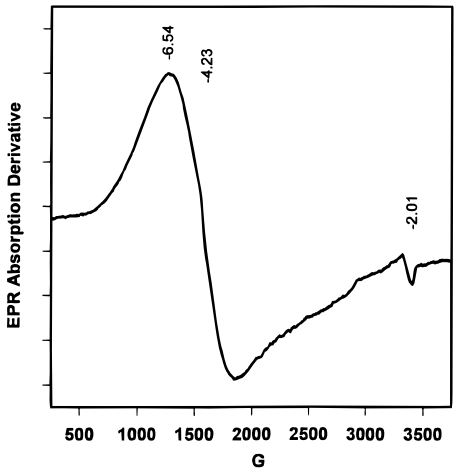

FIGURE 2: EPR spectrum of 410 μM Co(II)-substituted *B. fragilis* metallo-β-lactamase. Operating parameters were as follows: receiver gain, 3.2×10^4; modulation frequency, 100 kHz; modulation amplitude, 9.9 G; field center, 2000 G; sweep width, 3500 G; microwave frequency, 9.45 GHz; microwave power, 10 mW; number of scans, 5; temperature, 11 K.

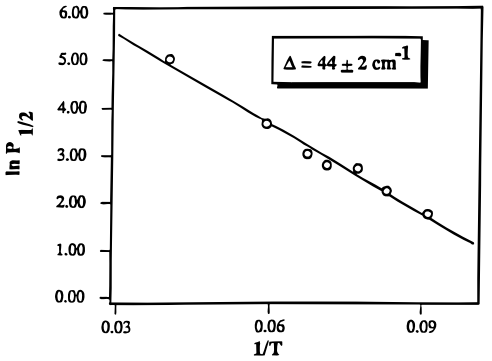

FIGURE 3: Plot of ln $P_{1/2}$ vs $1/T$. $P_{1/2}$'s were evaluated by plotting $S/\sqrt{P}$ vs log *P*. The line was drawn using a linear least-squares fit.

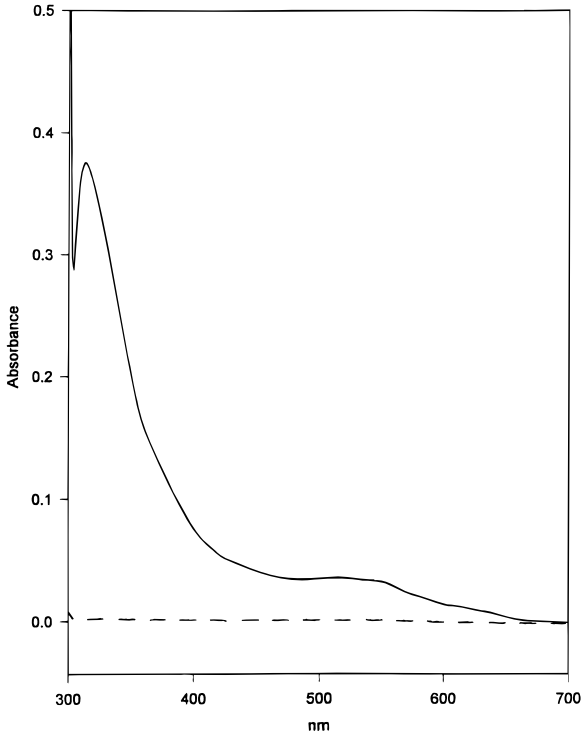

FIGURE 4: Solid line: UV−Vis difference spectrum of 200 μM Co(II)-substituted *B. fragilis* metallo-β-lactamase. Dashed line: UV−Vis difference spectrum of 200 μM Co(II)-substituted C168S mutant of the enzyme. Difference spectra were achieved by subtracting the spectrum of 200 μM Zn(II) enzyme from the spectra of the Co(II)-substituted enzymes. The spectra were collected at 25 °C on a Cary 1 UV−Vis spectrophotometer.

has disappeared (Figure 4, dashed line). This observation substantiates that Cys168 is actually bound to one of the metal ions in the Co(II)-substituted enzyme. The metal stoichiometry of the Co(II)-substituted and Zn(II)-containing C168S mutants is similar to that of the wild-type enzyme; however, there is a 10^3-10^4-fold decrease in k_{cat} and a significant increase in K_M values for the mutant proteins (Table 3), suggesting that Cys168 plays an important role in substrate binding and hydrolysis.

The weak features in Figure 4 between 550 and 650 nm are Laporte-forbidden Co(II) d−d transitions (Lever, 1984; Garmer & Krauss, 1993). The intensities of d−d transitions can be correlated with Co(II) coordination numbers (Elgren et al., 1994; Lever, 1984): in a four-coordinate Co(II) site, the extinction coefficient of the d−d transition is 300 $M^{-1}\cdot cm^{-1}$, as observed in Co(II)-substituted carbonic anhydrase (Figure 5a); in a five-coordinate Co(II) site, the extinction coefficient is 115 $M^{-1}\cdot cm^{-1}$; and in a six-coordinate Co(II) site, the extinction coefficient is 5−40 $M^{-1}\cdot cm^{-1}$. The intensities ($\epsilon_{550} \approx 30$ $M^{-1}\cdot cm^{-1}$) of the d−d transitions in the Co(II)-substituted *B. fragilis* β-lactamase (Figure 5b) suggest, as predicted by the temperature-dependent EPR experiments, that both Co(II)-binding sites are six-coordinate. This result marks another major difference between the *B. fragilis* and *B. cereus* enzymes since the catalytic Zn(II) of the *B. cereus* enzyme was reported to be five-coordinate by Waley and co-workers using MCD and X-ray structural studies (Bicknell et al., 1986; Sutton et al., 1987) and four-coordinate by Carfi et al. (1995). However, these same d−d transitions were not readily observed in the Co(II)-substituted C168S mutant (Figure 4). As sulfur donor ligands have been shown to enhance the molar absorptivity

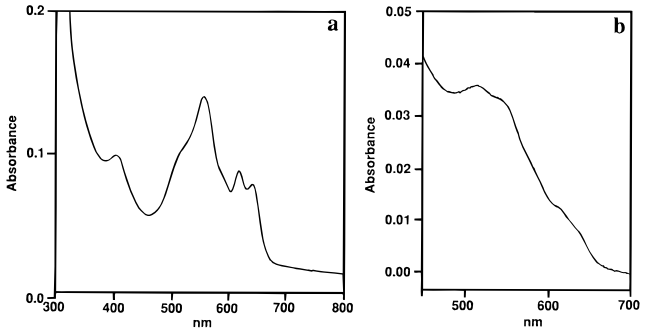

FIGURE 5: (a) d−d region of 500 μM Co(II)-substituted carbonic anhydrase and (b) enlargement of the 450−700 nm region of the Co(II)-substituted *B. fragilis* metallo-β-lactamase difference spectrum from Figure 4. Operating conditions were the same as described in Figure 4.

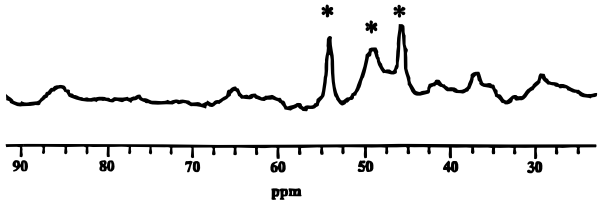

FIGURE 6: Downfield region of the ^{1}H NMR spectrum of 1 mM Co(II)-substituted *B. fragilis* metallo-β-lactamase at pH 7.4 with the three solvent-exchangeable signals marked with asterisks. The spectra were collected at 25 °C on a Bruker AMX-500. Acquisition parameters were the following: acquisition time, 65.5 ms; sweep time, 125 kHz; number of scans, 400 000. A line broadening of 50 Hz was used to process the spectra.

of six-coordinate Co(II) d−d transitions (Bertini & Luchinat, 1984; Lever, 1984), the molar absorptivity of the d−d transition in the Co(II)-substituted wild-type enzyme must have been enhanced by the cysteine sulfur ligation to the metal ion. The Cys ligand is presumably replaced by a Ser in the C168S mutant; thus, it is very likely that the molar absorptivity of the d−d transitions is diminished to a value not readily observable in the UV−Vis spectrum.

Nuclear Magnetic Resonance Spectroscopy. The above sequence comparison also implies that the *B. fragilis* enzyme might contain three histidine metal-binding ligands in the catalytic site. Since the paramagnetic Co(II) substitutes for the naturally-occurring Zn(II) in the *B. fragilis* β-lactamase to produce an active, viable enzyme, we used proton NMR spectroscopy to ascertain whether histidine ligands might serve as metal-binding ligands. Specifically, we examined the paramagnetically-shifted resonances of the Co(II)-substituted enzyme to establish whether any of them originated from the N−H protons of the bound histidine residues.

The proton NMR spectrum of the Co(II)-substituted enzyme between 20 and 90 ppm is shown in Figure 6. There are several possible paramagnetically-shifted resonances present in this chemical shift region; however, all of the observed peaks are very broad, probably due to an observed aggregation of the protein above 1 mM. Aggregation increases the effective size of the protein, decreases the rotational correlation time of the resonating nuclei, and, thus, increases the observed line widths of the NMR signals (Bertini & Luchinat, 1986). Several experiments (ionic strength changes, adding detergents, and changing pH, etc.) to alleviate this aggregation were attempted, but all were unsuccessful.

Nonetheless, there were at least three proton NMR signals at 46, 49, and 54 ppm that disappeared when switching the protein sample into D_2O buffer; thus, they arise from solvent-exchangeable protons in the protein. The proton NMR spectra of $Co(Me\text{-}Im)_2Cl_2$, $Co(Im)_2Cl_2$, and $Co(4\text{-}Me\text{-}Im)_2Cl_2$ model complexes have established that the exchangeable N−H protons of imidazoles are shifted into the chemical shift region of 45−65 ppm by high-spin Co(II) (Bertini et al., 1981). Therefore, the three proton NMR signals at 46, 49, and 54 ppm observed in the NMR spectrum (Figure 6) are very likely due to the N−H protons of the imidazole rings of the histidine ligands bound to the Co(II) ion(s) in the enzyme, providing direct evidence that there are three histidine ligands in the metal-binding sites of the Co(II)-substituted β-lactamase from *B. fragilis*. The NMR characteristics of Co(II)-substituted β-lactamase are also very similar to those of Co(II)-substituted carbonic anhydrase at neutral pH (Bertini et al., 1993). By analogy to the results obtained for the *B. cereus* enzyme (Bicknell et al., 1986; Sutton et al., 1987; Carfi et al., 1995), we conclude that the metallo-β-lactamase from *B. fragilis* has three histidine ligands (His86, -88, and -149 or -210) in its metal-binding sites and the three His residues are bound to the same metal ion.

Site-Directed Mutagenesis Studies. So far, we have been able to identify three His ligands and one Cys ligand, but more ligands are needed to complete the coordination shells of the two metal-binding sites of *B. fragilis* metallo-β-lactamase. Carboxylate ligands to Co(II) or Zn(II) are very common in metalloproteins and metalloenzymes, but they are very difficult to detect using common spectroscopic techniques such as EPR and UV−Vis spectroscopies. The broadness of the paramagnetically-shifted proton NMR resonances we obtained on the enzyme (Figure 6) precluded the possibility of observing resonances of carboxylate ligands [for example, $-CH_2-$ resonances were expected between 30 and 60 ppm in a Co(II) protein (Bertini et al., 1993)]. Therefore, we attempted to discern possible carboxylate ligands in the metal-binding sites of *B. fragilis* metallo-β-lactamase using site-directed mutagenesis. Since there are no conserved glutamic acid residues present in group 3 β-lactamases (Rasmussen et al., 1990; Carfi et al., 1995), aspartic acid residues were chosen for study in this work.

Comparison of the sequences of the *B. fragili*s and *B. cereus* enzymes suggested that one of the aspartic acid residues (Asp90) in the weaker metal-binding site of the *B. cereus* enzyme is conserved in the *B. fragilis* enzyme (Rasmussen et al., 1990). Actually, Asp90 is the only aspartic acid residue retained in all five reported sequences of the group 3 β-lactamases (Carfi et al., 1995). The substitution of Asp90 with valine resulted in a D90V mutant that binds 0.43 mol of Zn(II) per mole of enzyme, exhibits a 5-fold decrease in substrate affinity, and has a k_{cat} 6400-fold less than the wild-type (Table 3). Apparently, Asp90 is a ligand to Zn(II), and removal of this ligand disrupts the binding affinity for both metal ions. Since another aspartic acid residue (Asp81) in the weaker metal-binding site of the *B. cereus* enzyme (Sutton et al., 1987) is not conserved in the *B. fragilis* enzyme, site-directed mutations were made of other conserved aspartic acid residues [the complete X-ray crystallographic structure at 2.5 Å (Carfi et al., 1995) is not available to view at present].

Position 152 contains an aspartic acid conserved in four of the five known sequences of group 3 β-lactamases (Carfi et al., 1995). The D152V mutant, much like the D90V mutant, has a metal stoichiometry of less than 1 mol of Zn(II) per mole of enzyme (Table 3), indicating that the metal-binding sites have also been perturbed due to the mutation. The D152V mutant has a k_{cat} 8100-fold smaller than wild type, suggesting that this aspartic acid residue is important catalytically and might be a metal-binding ligand. However, this mutation probably does not result in a large structural change since its K_M, indicative of substrate binding, is unchanged (Table 3).

The other aspartic acid residue which is conserved in four of the five known sequences of group 3 β-lactamases is Asp56 (Carfi et al., 1995). The conversion of Asp56 to a valine does not change the metal−enzyme stoichiometry and K_M, but decreases the relative k_{cat} by *ca.* 1000-fold. Therefore, Asp56 is probably not involved in metal-binding, but might play an important role in catalysis.

Finally, Asp61 and Asp183, which are conserved in the group 3 β-lactamases from *B. cereus* and *B. fragilis*, were mutated to valines; in these cases, no more than one Zn(II) atom was bound, and the K_M and k_{cat} were marginally changed (Table 3). The decrease in k_{cat} of the D61V and D183V mutants closely approximates the expected decrease in catalytic activity for β-lactamases with this Zn(II) content (Figure 1 and Table 3). Thus, the loss of a single Zn(II) is not catastrophic for β-lactamase activity, since the effect of a second metal is to increase k_{cat} by only a factor of 2.

The interpretation of the mutagenesis data is complicated by the fact that Zn(II) is incorporated into the protein during the procedures for resolubilization of the inclusion bodies through a denaturation−renaturation cycle. The changes in Zn(II) content found for the mutant proteins may reflect influence by the mutation in the kinetic competition between refolding and metal ion binding. Presuming that is not the case, then only Asp61 and Asp183 retain Zn(II) at a content that correlates with a predictable loss in enzyme activity and are ligands important in the binding of Zn(II) but not in its active-site placement. Mutant proteins D90V and D152V reveal multiple effects since the decrease in k_{cat} far exceeds the expected 70−80% reduction in k_{cat} for a β-lactamase with this Zn content. Their exact role is not clear at present; a working hypothesis views Asp90 and Asp152 as essential for the optimal positioning of the residual Zn(II) ion for catalysis possibly achieved through their bridging of both metal sites. However, this hypothesis is not supported by the data from the EPR studies of the Co(II)-substituted enzyme, although the latter may differ subtly from the Zn(II) wild-type enzyme. Asp56 is not a ligand to either Zn(II) ion.

All the observations from this work suggest that the group 3 β-lactamase from *B. fragilis* has two metal-binding sites. EPR and UV−Vis studies with the Co(II)-substituted enzyme are consistent with a five/six-coordinated site for both metal ions. We postulate that one site contains three histidine and one cysteine ligand to the metal ion, reminiscent of the solvent-accessible catalytic site in the *B. cereus* enzyme. It is possible that the remaining ligand(s) in this site may be water molecule(s), one of which may serve as a nucleophile during the catalysis (Scheme 1). For the second metal ion, Asp61, Asp90, Asp152, and Asp183 are possible ligands. At present, we cannot rule out the possibility of other amino

acids (such as serine, threonine, and lysine) and H_2O acting as ligands in addition to the aspartic acid carboxylates at this site.

CONCLUSIONS

Atomic absorption studies on the native, Zn(II)-containing protein and on the Co(II)-substituted protein, coupled with spin integration of the EPR signal of the latter, are consistent with the β-lactamase from *B. fragilis* containing 2 mol of tightly bound metal ions per mole of enzyme. The EPR studies of the Co(II) enzyme demonstrated that the two metal ions are not spin-coupled and that both metal ions are probably five/six-coordinated, which is confirmed by the magnitude of the extinction coefficients of the Co(II) d−d transition bands in the UV−Vis spectrum of the Co(II)-substituted enzyme. UV−Vis studies also revealed the presence of a S-to-Co(II) LMCT transition, suggesting that a cysteine residue, presumably Cys168, serves as a ligand to one of the metal ions. This is supported by the results obtained from the C168S mutant of the enzyme. Proton NMR studies revealed that there are three histidine residues, presumably His86, -88, and -149 or -210, as metal ligands. All these results indicate that the β-lactamase from *B. fragilis* has one metal-binding site similar to the catalytic site of the enzyme from *B. cereus*. Site-directed mutagenesis studies identified Asp61, Asp90, Asp152, and Asp183 as possible ligands to the metal ion at the second site. The loss of Asp90 and Asp152 also decrease k_{cat} 6400−8100-fold, far in excess of the expected loss of the enzyme activity due to the loss of the Zn(II) content, suggesting important roles for these carboxylates in the catalytic cycle.

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