Kinetic Properties and Metal Content of the Metallo-β-lactamase CcrA Harboring Selective Amino Acid Substitutions*

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The crystal structure of the metallo-β-lactamase CcrA indicates that the active site of this enzyme contains a binuclear zinc center. To aid in assessing the involvement of specific residues in β-lactam hydrolysis and susceptibility to inhibitors, individual substitutions of selected amino acids were generated. Substitution of the zinc-ligating residue Cys104 with Ser (C181S) resulted in a significant reduction in hydrolytic activity; kcat values decreased 2–4 orders of magnitude for all substrates. Replacement of His99 with Asn (H99N) significantly reduced the hydrolytic activity for penicillin and imipenem. Replacement of Asp103 with Asn (D103N) showed reduced hydrolytic activity for cephaloridine and imipenem. Deletion of amino acids 46–51 dramatically reduced both the hydrolytic activity and affinity for all β-lactams. The metal binding capacity of each mutant enzyme was examined using non-denaturing electrospray ionization mass spectrometry. Two zinc ions were observed for the wild-type enzyme and most of the mutant enzymes. However, for the H99N, C181S, and D103N enzymes, three different zinc content patterns were observed. These enzymes contained two zinc molecules, one zinc molecule, and a mixture of one or two zinc molecules/enzyme molecule, respectively. Two enzymes with substitutions of Cys104 or Cys104 and Cys155 were also composed of mixed enzyme populations.

Metallo-β-lactamases require zinc or another metal cofactor to be enzymatically active and capable of hydrolyzing the amide bond of the β-lactam ring (1–5). The metallo-β-lactamase CcrA hydrolyzes almost all known β-lactams and is not effectively inactivated by the marketed β-lactamase inhibitors clavulanic acid, sulbactam, and tazobactam (6–8). The crystal structure of the metallo-β-lactamase CcrA3 indicates that the active site of this enzyme is at the edge of the β-sandwich of the four-layer α/β/α/α molecule and contains two zinc atoms (1). Zn1 is coordinated to four ligands in a tetrahedral geometry: His59, His101 and His162, and a solvent molecule defined as water 1 (Wat1).1 Zn2 ligates with three amino acids (Asp103, Cys181, and His223) and two water molecules (Wat1 and Wat2) (Fig. 1). The proposed mechanism of CcrA enzymatic hydrolysis includes formation of a hydroxide ion from Wat1. This hydroxide ion mounts a nucleophilic attack on the carbonyl carbon atom of the β-lactam. Atomic absorption studies of the metal content of CcrA (9) confirmed that CcrA binds two atoms of Zn2+ and that the presence of both metal ions is required for full catalytic activity.

In this work, we describe a set of CcrA derivatives harboring selected amino acid substitutions of active-site and nearby residues (see Table I) and the involvement of these individual amino acid residues in enzymatic activity. Purified mutant proteins were used to analyze the β-lactam hydrolytic and inhibition properties conferred by each amino acid substitution. The metal content of the CcrA wild-type and mutant enzymes was determined using a novel approach, electrospray ionization mass spectrometry (ESI/MS) utilizing non-denaturing- and denaturing conditions, and was then compared with the measurements obtained by a classic approach, atomic absorption spectrophotometry (AAS). The effects of the specific amino acid substitutions on metal content and catalytic activity were analyzed.

EXPERIMENTAL PROCEDURES

Bacterial Strains—Escherichia coli strains DH5α and BL21 (DE3) (10) were used for gene construction and protein production, respectively.

Antibiotics and Reagents—Penicillin G and 1,10-phenanthroline were purchased from Sigma. Other reagents were obtained as indicated: cephaloridine from Lilly, imipenem from Merck, tazobactam from Lederle Laboratories, nitrocefin from Becton Dickinson Microbiology Systems (Hunt Valley, MD), and BRL 42715 from SmithKline Beecham (Worthing, United Kingdom).

Site-directed Mutagenesis—All ccrA codon changes were introduced into the respective genes ccrA1 and ccrA3 using modified polymerase chain reaction (PCR) techniques employing T7G polymerase (11). The enzymes CcrA1 and CcrA3 are both naturally occurring Bacteroides fragilis metallo-β-lactamases that differ at three amino acids, none of which is located within the active-site cavity (10). For the Cys104 to Ser (C104S) and Cys104 to Val (C104V) mutations, two sets of primers were used to amplify the ccrA1 gene as overlapping 5′-terminal and 3′-terminal regions and 3′-carboxyl-terminalcoding fragments with the desired mutation at the 3′ or 5′ end, respectively. The amino-terminal and carboxyl-terminal coding PCR products were melted, mixed, annealed, and amplified a second time to give full-length ccrA1 containing the desired mutation. These two mutations were constructed sequentially beginning with the C104V encoding mutation (12). The remaining mutations were constructed using ccrA3 as the template gene (10). For the Cys181 to Ser (C181S), Lys184 to Arg (K184R), and Asn193 to Asp (N193D) mutations, one oligonucleotide was constructed that covered the region encoding the codon to be changed and harboring the new codon. A second oligonucleotide covering the 3′ terminal region of the gene was used as the partner primer in the first PCR. A short 3′ double-stranded DNA fragment was produced. This 250–300-base pair double-stranded DNA fragment was used as one primer in combination with a 5′ primer in the second PCR to produce the carboxyl-terminal 650 base pairs of the gene. The resulting PCR product was cloned into pUC119. A fragment from this clone was used to replace the carboxyl-terminal region of ccrA, from the internal KpnI site to the 3′ end, into a pGEM expression vector containing a ccrA gene minus the signal sequence (6, 10, 12). The His99 to Asn (H99N), Asp103 to Asn (D103N), Cys104 to Arg (C104R), and Δ46–51 mutations were constructed in a similar manner, except that

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† The abbreviations used are: Wat, water; ESI/MS, electrospray ionization mass spectrometry; AAS, atomic absorption spectrophotometry; PCR, polymerase chain reaction; PenG, penicillin G; CLD, cephaloridine; IMP, imipenem.
the amino-terminal region of the gene was constructed first and used in combination with a carboxyl-terminal primer in the second PCR to create the full-length gene. The DNA sequence of all PCR products and the presence of the expected mutation were confirmed by DNA sequence analysis.

Enzyme Purification—All CcrA-encoding genes were under transcriptional and translational control of a T7 promoter and expressed in E. coli BL21 (DE3) cells from a kanamycin resistance-conferring vector. Purification of all enzymes was performed as described (6). Briefly, 2 liters of mid-log phase cells were induced by the addition of 1 mM (final concentration) isopropyl-β-D-thiogalactopyranoside for 2 h. Overproduction of the β-lactamase resulted in the sequestering of the β-lactamase into inclusion bodies. The inclusion bodies were harvested following disruption of the expressing cells using a French press (15,000 p.s.i.) and centrifugation. Two ESI mass spectra were obtained for each protein under acetate buffer prior to ESI/MS analysis by dilution and centrifugal filtration. Material that precipitated during dialysis was removed by centrifugation. Protein concentrations were determined using the BCA assay (Pierce).

Enzyme Kinetics and Inhibition Study—Hydrolysis rates for the putative enzymes were determined in 10 mM HEPES, pH 7.2, using a Beckman DU7400 or Gilford 250 spectrophotometer. The kinetic parameters $k_{cat}$ and $K_m$ were derived from the initial velocities obtained from six to eight substrate concentrations using the computer program ENZPACK (Biosoft/Elsevier). These parameters are presented as the mean value of at least duplicate experiments with the S.D. for each parameter being <20%. For the inhibition studies, enzyme and inhibitor were preincubated at 25 °C in a volume of 50 μl for 10 min before the addition of 50 μg/ml nitrocefin (final volume of 1000 μl). $IC_{50}$ values were determined graphically.

Metal Content Determination Using ESI/MS—Samples of the CcrA wild-type and mutant enzymes were exchanged with 10 mM ammonium acetate buffer prior to ESI/MS analysis by dilution and centrifugal filtration. Two ESI mass spectra were obtained for each protein under native state and denatured conditions. Denaturation of the proteins was performed by adding 40 μl of a 50:50 (v/v) water/acetonitrile solution containing 0.4% formic acid to 20 ml of a 50:50 (v/v) water/acetonitrile solution containing 0.4% formic acid to 20 μl of the native protein in 10 mM ammonium acetate. The native or denatured protein solutions (0.5–3.0 nmol) were introduced into the ESI mass spectrometer at a flow rate of 3–5 μl/min using a Harvard Model PHD 2000 syringe infusion pump. All mass spectra were acquired using an API 365 triple quadrupole mass spectrometer equipped with an atmospheric pressure ionization source and a PE-Sciex ionspray (pneumatically assisted electrospray) interface. A Power Macintosh 9600/300 computer was used for instrument control, data acquisition, and data processing. A mild set of ESI interface conditions was employed for the detection of noncovalent metal-protein complexes, i.e. low curtain gas (desolvation) pressure and a low orifice (declustering) potential (13–15). High purity nitrogen gas was used as the nebulizing gas. The mass scale of the spectrometer was calibrated with polypropylene glycol. The mass resolution of the spectrometer was tuned to give a constant peak width of 1 Da across the mass range of interest. Full scan mass spectra were acquired using a step size of 0.2 Da with a scan time of 7.5 s across the m/z range of 2000–3000 for the native protein samples and with a scan time of 6.8 s across the m/z range of 30–3000 for the denatured protein samples. Typically, 20–40 scans were acquired and added to yield a mass spectrum. Protein molecular masses were obtained by deconvoluting the multiply charged protein mass spectra using the PE-Sciex hyper mass program. The zinc content of each protein was derived from the mass difference between the native and denatured proteins.

Determination of Zinc Content Using Atomic Absorption Spectrophotometry—The wild-type and amino acid-substituted enzymes were dialyzed against zinc-free 10 mM HEPES, pH 7.2, at 4 °C with multiple changes of dialysis buffer to remove zinc from the sample buffer. Protein concentrations of the dialyzed samples were determined using the BCA assay and adjusted to a final concentration of 1 mg/ml. The metal content values reported for each sample are an average of readings from two independent experiments.

RESULTS

Construction of Amino Acid Substitutions and Enzyme Preparation—Using the described PCR techniques, eight mutant genes were successfully constructed and confirmed by DNA sequence analysis (Table I). These mutant genes were placed into a T7 expression plasmid by replacing the carboxyl-termi-

![Fig. 1. Stereoscopic representation of the atomic model of the active site of CcrA3. The zinc- and metal-bound solvent molecules are depicted.](image-url)
nal region of a ccrA gene lacking a signal sequence that was already present in the vector. The protein produced following isopropyl-β-D-thiogalactopyranoside induction accumulates in inclusion bodies, from which it is easily solubilized and refolded (6). To ensure proper refolding, the enzymes were refolded in a 30-fold excess of Zn2+. As an assessment of proper folding, circular dichroism studies were performed on selected enzymes. These studies revealed no structural differences when compared with the wild-type enzyme (data not shown). The enzymatic activity of the refolded enzymes paralleled the in vivo activity of the same constructs. These observations strongly suggest that the enzymes used in these studies have correctly and efficiently refolded. The purity and molecular mass of all constructed proteins were confirmed by SDS-polyacrylamide gel electrophoresis and ESI/MS.

### Kinetic Properties and Zn2+ Binding of CcrA Derivatives

**TABLE II**

| Protein       | Penicillin G | Cephaloridine | Imipenem |
|---------------|--------------|---------------|----------|
|               | hcat | Km | hcat/Km | hcat | Km | hcat/Km | hcat | Km | hcat/Km |
| CcrA2a         | 94   | 25  | 3.8     | 3.0   | 0.0018 | 73   | 12   | 6.1   | 74   | 140 | 0.52 |
| H99N          | 0.28 | 150 | 0.00018 | 70   | 0.026 | 700  | 0.0024 | 1.0   | 180  | 0.00016 |
| C181S         | 0.65 | 100 | 0.0009  | 55   | 0.0034 | 700  | 0.00014 | 3.7   | 440  | 0.00016 |
| D103N         | 50   | 310 | 0.0077  | 17   | 0.016 | 46   | 1.4   | 3.2   | 2.6   | 4.2   | 0.00016 |
| Δ46–51        | 46   | 260 | 0.063   | 46   | 1.4   | 46   | 3.6   | 3.2   | 2.6   | 0.00016 |
| K184R         | 80   | 190 | 0.0009  | 33   | 1.4   | 120  | 3.6   | 3.2   | 2.6   | 0.00016 |
| N193D         | 790  | 930 | 0.85    | 330  | 7.6   | 700  | 0.0035 | 0.3   | 140  | 0.00016 |
| CcrA1b        | 190  | 40  | 4.8     | 42   | 7.4   | 200  | 7.4   | 0.3   | 140  | 0.00016 |
| C104S/C155V   | 23   | 55  | 0.43    | 58   | 8.9   | 6.1  | 3.2   | 2.0   | 0.74  | 9.5   | 0.00016 |

* Data from Rasmussen et al. (7).

* Data from Yang et al. (6).

### Hydrolytic Activity of Mutant Enzymes for the Major β-Lactam Classes

The catalytic activities of the mutant enzymes were compared with their respective wild-type enzymes for penicillin G (PenG), cephaloridine (CLD), and imipenem (IMP) (Table II). In general, substitution of amino acid residues that ligate to one of the two zinc molecules (C104R, D103N, or C181S) resulted in the most significant change in enzymatic activity. Substitution of the Zn2-chelating residue Cys181 with Ser resulted in a significant reduction (greater than 2–3 orders of magnitude) in hydrolytic activity for PenG, CLD, and IMP (Table II). The affinity of the C181S enzyme for CLD and IMP was reduced 2–3-fold. For PenG, the affinity was reduced 20-fold. For the K184R mutant, the affinity was reduced 7-fold. The loop mutant (deletion of amino acids 46–51, removing the disordered loop) showed a dramatic reduction in both hydrolytic activity and affinity for all the β-lactam substrates. The most significant change observed was for CLD, with a reduced physiological efficiency (hcat/Km) of 4500-fold compared with the wild-type enzyme (Table II). Substitution of Lys184 with Arg and Asn resulted in increased hcat and Km values for PenG. Substitution of Cys181 with Arg had the least effect on the hcat values for PenG and CLD, with a slight effect on the hcat for IMP. The double amino acid substitution C104S/C155V showed significantly increased affinity for IMP.

### Inhibition Profiles of Mutant Enzymes

**TABLE III**

**Inhibition profile of CcrA β-lactamase and mutant proteins**

| Enzyme          | Tazobactam | BRL 42715 | 1,10-Phenanthroline |
|-----------------|------------|-----------|---------------------|
| CcrA3           | 300        | 0.05      | 15                  |
| H99N            | 700        | 180       | 2                   |
| C181S           | 1800       | >2000     | 3.5                 |
| D103N           | 550        | 0.36      | 50                  |
| Δ46–51          | >2500      | 420       | 45                  |
| C104R           | 460        | 0.25      | 50                  |
| K184R           | 330        | 0.008     | 16                  |
| N193D           | 750        | 0.22      | 9.5                 |
| CcrA1           | 400        | 0.09      | 28                  |
| C104S/C155V     | 220        | 0.007     | 9.5                 |

**Inhibition of CcrA Derivatives**

**TABLE IV**

**Kinetic parameters for CcrA and selected amino acid-substituted enzymes**

| Protein       | Penicillin G | Cephaloridine | Imipenem |
|---------------|--------------|---------------|----------|
|               | hcat | Km | hcat/Km | hcat | Km | hcat/Km | hcat | Km | hcat/Km |
| CcrA2a         | 94   | 25  | 3.8     | 3.0   | 0.0018 | 73   | 12   | 6.1   | 74   | 140 | 0.52 |
| H99N          | 0.28 | 150 | 0.00018 | 70   | 0.026 | 700  | 0.0024 | 1.0   | 180  | 0.00016 |
| C181S         | 0.65 | 100 | 0.0009  | 55   | 0.0034 | 700  | 0.00014 | 3.7   | 440  | 0.00016 |
| D103N         | 50   | 310 | 0.0077  | 17   | 0.016 | 46   | 1.4   | 3.2   | 2.6   | 0.00016 |
| Δ46–51        | 46   | 260 | 0.063   | 46   | 1.4   | 46   | 3.6   | 3.2   | 2.6   | 0.00016 |
| K184R         | 80   | 190 | 0.0009  | 33   | 1.4   | 120  | 3.6   | 3.2   | 2.6   | 0.00016 |
| N193D         | 790  | 930 | 0.85    | 330  | 7.6   | 700  | 0.0035 | 0.3   | 140  | 0.00016 |
| CcrA1b        | 190  | 40  | 4.8     | 42   | 7.4   | 200  | 7.4   | 0.3   | 140  | 0.00016 |
| C104S/C155V   | 23   | 55  | 0.43    | 58   | 8.9   | 6.1  | 3.2   | 2.0   | 0.74  | 9.5   | 0.00016 |
**DISCUSSION**

The metallo-\(\beta\)-lactamases CcrA1 and CcrA3 from *B. fragilis* have a broad substrate profile and are refractory to inhibition by the commercially available \(\beta\)-lactamase inhibitors (6, 7, 16). These enzymes require a metal cofactor to be enzymatically active (2, 3). Within the active site, there are two zinc ligands, designated Zn1 and Zn2 (1). Using site-directed mutagenesis, eight different ccrA genes have been constructed that encode enzymes with amino acid substitutions in or near the active site. The hydrolytic activity, susceptibility to inhibitors, and metal content of each mutant enzyme were investigated. The results indicate that many of the substituted amino acids play a critical role in the enzymatic function of the protein. The most dramatic effects were observed when the enzyme harbored an amino acid substitution of one of the zinc-ligating residues.

H99N, D103N, and C181S. Two \(\text{Zn}^{2+}\) atoms/enzyme molecule were observed for H99N. For C181S, only one \(\text{Zn}^{2+}\) atom/ enzyme molecule was observed. The D103N enzyme preparation was composed of a mixed population with either one or two zinc molecules/enzyme molecule. The major component for the D103N enzyme, corresponding to 71% of the enzyme population, held two \(\text{Zn}^{2+}\) atoms/enzyme molecule, and 29% of the enzyme content held only one \(\text{Zn}^{2+}\) atom. For C104R, 32% of the native enzyme bound two \(\text{Zn}^{2+}\) molecules/enzyme molecule, and 68% bound one \(\text{Zn}^{2+}\) molecule. For the double mutant C104S/C155V, in which two enzyme populations containing two \(\text{Zn}^{2+}\) atoms (83%), and the remaining enzyme harbored one zinc atom. Substitutions of two other residues (K184R and N193D) as well as the loop deletion did not result in altered \(\text{Zn}^{2+}\) content. These mutant enzymes contained two \(\text{Zn}^{2+}\) atoms/enzyme molecule.

The metal content of the enzymes as measured by AAS is reported in Table IV. The zinc content values reported by AAS generally corresponded well with the ESI/MS data. However, for H99N, only one \(\text{Zn}^{2+}\) atom/molecule of protein was observed by AAS, whereas ESI/MS data clearly indicated the presence of two zinc atoms/enzyme molecule under native conditions. The reported zinc content of CcrA3 by AAS was 1.41 \(\text{Zn}^{2+}\) atom/molecule of protein.

Among the most exciting findings of this study are the zinc content determinations for the wild-type and mutant enzymes. Two different methods were used to analyze the zinc content: ESI/MS and AAS. For most of the enzymes, the zinc content was comparable using either of the two methods. However, AAS tended to underestimate the zinc content of the enzyme relative to that observed by ESI/MS. This may be the result of the dialysis procedure that was required when performing AAS. Since this method cannot discriminate between enzyme-bound zinc and zinc in buffer solution, dialysis of the enzyme sample to remove extraneous \(\text{Zn}^{2+}\) was required. Unfortunately, this process may also remove weakly bound \(\text{Zn}^{2+}\) from the active site of the enzyme, resulting in an underestimated \(\text{Zn}^{2+}\) content. This is likely to be the case for the H99N enzyme. ESI/MS identified two zinc molecules/enzyme molecule, whereas only one zinc molecule/enzyme molecule was determined using AAS. This suggests that the H99N enzyme binds Zn1 with much less affinity than the wild-type enzyme and that this zinc is lost during dialysis. The lower IC\(_{50}\) value of H99N for the \(\text{Zn}^{2+}\) chelator 1,10-phenanthroline (2 \(\mu\)M) compared with that of CcrA3 (15 \(\mu\)M) supports the above hypothesis. Another unique advantage of using ESI/MS to measure \(\text{Zn}^{2+}\) content is the ability to detect multiple enzyme populations that have different \(\text{Zn}^{2+}\) contents within the same sample. This is evident in the ESI/MS data for D103N, C104R, and C104R/C155V, in which two enzyme populations containing one or two zinc molecules/enzyme molecule were observed. This novel and powerful application of ESI/MS has not been applied previously in studies of metallo-\(\beta\)-lactamases.

The substitution of the Zn2-coordinating residue Cys\(^{181}\) with Ser resulted in dramatic changes in enzymatic function with reduced hydrolytic activity for all the substrates tested (Table II) and reduced susceptibility to the inhibitor BRL 42715 (Table III). ESI/MS indicated the presence of only one zinc atom/molecule of C181S (Fig. 2, c and d). This might result from the substitution of a negatively charged cysteine sulfurl with a serine hydroxyl or the inability of the two remaining ligands...
(Asp\textsuperscript{103} and His\textsuperscript{223}) to capture a Zn\textsuperscript{2+} atom in the absence of the binding contribution of Cys\textsuperscript{181}. Recent x-ray crystallographic data (17) indicated that, although the overall structure of the C181S enzyme remains the same as the wild-type enzyme, with the side chain of Ser\textsuperscript{181} occupying the same spatial position as that of Cys\textsuperscript{181}, there is no electron density at the position of Zn2. Furthermore, in C181S, the distance between the remaining Zn1 and Wat1 was extended. It has been proposed that Wat1 exists as a hydroxide ion in the wild-type enzyme and is responsible for carrying out the nucleophilic attack on the \(\beta\)-lactam ring. Based on the changes in the Wat1 coordination, it is unlikely that Wat1 exists as a hydroxide ion in the C181S enzyme (17). This is likely to be the major factor affecting the catalytic activity of this enzyme. Interestingly, studies of the \textit{Bacillus cereus} metallo-\(\beta\)-lactamase II (2, 18) demonstrate the mono-zinc form of the enzyme to be nearly as active as the di-zinc form.

Another substitution that affects Zn2 binding is the replacement of Asp\textsuperscript{103} with Asn. ESI/MS data indicated that 29% of the D103N molecules harbored one zinc atom, whereas 71% harbored two zinc atoms (Fig. 2, a and f; and Table IV). However, the enzymatic activity of D103N was \(<\)71% that of CcrA3. Thus, the reduced hydrolytic activity of CLD and IMP and the decreased affinity for PenG cannot be fully accounted for by assuming that the di-zinc form of the enzyme is fully active. The carboxylate group of Asp\textsuperscript{103} is predicted to function as a general base during hydrolysis (17), forming interactions with both Zn2 and Wat1. The decreased enzymatic activity may more accurately reflect the inability of Asn to function as a general base to activate Zn2 and Wat1 as effectively as Asp. Substitution of Asp\textsuperscript{103} with Val has been reported by Crowder et al. (9). For this enzyme, the metal content (determined using AAS) was reported to be 0.43 ± 0.09. This is significantly less than the 1.2 zinc molecules/enzyme molecule observed for the D103N enzyme using AAS, suggesting that the valine-substituted enzyme is less effective at incorporating zinc into the active site than the Asn substitution. The \(k_{cat}\) value for the Val enzyme, using the cephalosporin nitrocefin as a substrate, decreased 6400-fold. Similarly, the \(k_{cat}\) value for the Asn enzyme, using cephaloridine as a substrate, showed a 260-fold decrease. In contrast to the cephaloridine \(k_{cat}\) value, only a 2-fold decrease in the \(k_{cat}\) value for penicillin G was observed for the D103N enzyme. This minor effect suggests that the main function of Asp\textsuperscript{103} is to coordinate and activate Zn2 and that it plays no direct role in the catalytic cycle as might be predicted, and has been proposed, from the nitrocefin hydrolysis data (9). Substitution of Asp\textsuperscript{103} clearly has an additional effect on cephalosporin hydrolysis beyond the decreased zinc binding. Both the Val and Asn substitutions may affect the active-site architecture such that cephalosporin hydrolysis is more dramatically affected than the hydrolysis of other classes of \(\beta\)-lactams.

His\textsuperscript{99} is one of the three histidine residues that ligate to Zn1 (Fig. 1). Kinetic and inhibition data (Tables II and III) indicated that His\textsuperscript{99} plays an active role in enzymatic function.

**FIG. 2.** ESI mass spectra of CcrA3, C181S, and D103N. a, native CcrA3; b, denatured CcrA3; c, native C181S; d, denatured C181S; e, native D103N; f, denatured D103N. The molecular masses for each protein under native and denatured conditions are indicated. amu, atomic mass units.
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since the physiological efficacy of H99N was reduced 2000–3000-fold for PenG and IMP and >200-fold for CLD. Interestingly, H99N also had a 29-fold higher hydrolytic activity for nitrocefin (a chromogenic cephalosporin) than PenG (data not shown). This indicated that some cephalosporins might fit in the structure of H99N better than five-membered ring β-lactams. As discussed previously, two zinc molecules/enzyme molecule was observed using ESI/MS, whereas AAS identified only one zinc molecule/enzyme molecule. The double hydrolytic activity for PenG (5.9-fold) than for IMP, compared to 1.2-fold difference for CcrA3 (Table II). The double hydrolytic activity for PenG (5.9-fold) than for IMP. The same pattern was also seen for the CcrA3 H99N mutant enzyme.

Although not directly ligated to either zinc molecule, substitutions of Cys\(^{104}\) (C104R and C104S/C155V) resulted in proteins harboring either one or two zinc atoms/enzyme molecule. The location of Cys\(^{104}\) within the enzyme might play a critical role in this phenomenon. Cys\(^{104}\) is at the active site and close to both zinc molecules (Fig. 1). The sulphydryl group on the cysteine might contribute more stability to the protein structure than either arginine or serine. Residue 104 is an arginine in the B. cereus β-lactamase II (20). Comparing the substrate profile for this enzyme (21), the \(k_{\text{cat}}\) value for PenG was 3.8-fold higher than that for IMP. The C104R enzyme also showed stronger hydrolytic activity for PenG (5.9-fold) than for IMP, compared with a 1.2-fold difference for CcrA3 (Table II). The double mutant C104S/C155V contains the same residues at positions 104 and 155 as the metallo-β-lactamase IMP-1 from Serratia marcescens (22). IMP-1 has stronger hydrolytic activity for PenG than for IMP. The same pattern was also seen for the C104S/C155V enzyme. The reduced hydrolytic activity of β-lactamase II and IMP-1 for CLD (22, 23) was not observed in either of the CcrA Cys\(^{104}\) substitutions and may relate to differences in other regions of these enzymes.

Amino acids 46–51 form a “disordered loop” in CcrA3 (1). Removal of these six amino acids significantly affected the activity against PenG and CLD, the predominant effect being on substrate affinity (increased \(K_m\)) (Table II). Activity against IMP and susceptibility to BRL 42715 were only marginally affected. The crystallographic structure of CcrA3 indicates that residues 46–51 compromise the end of a hairpin loop that protrudes into the solvent (1). This “flap” could adjust upon substrate binding to accommodate a variety of β-lactam side chain substitutions (1). Removal of the loop may restrict substrate specificity, resulting in reduced affinity for many β-lactams.

The data presented were obtained using the powerful technique of ESI/MS to assess the Zn\(^{2+}\) content and its relation to enzymatic activity. The Zn\(^{2+}\) content was differentially affected by various amino acid substitutions. From these analyses, it becomes clear that the zinc content was not the only factor contributing to the observed changes in the enzymatic activity of these mutant enzymes.

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