Metal Binding Asp-120 in Metallo-β-lactamase L1 from Stenotrophomonas maltophilia Plays a Crucial Role in Catalysis*

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Metallo-β-lactamase L1 from Stenotrophomonas maltophilia is a dinuclear Zn(II) enzyme that contains a metal-binding aspartic acid in a position to potentially play an important role in catalysis. The presence of this metal-binding aspartic acid appears to be common to most dinuclear, metal-containing, hydrolytic enzymes; particularly those with a β-lactamase fold. In an effort to probe the catalytic and metal-binding role of Asp-120 in L1, three site-directed mutants (D120C, D120N, and D120S) were prepared and characterized using metal analyses, circular dichroism spectroscopy, and pre-steady-state and steady-state kinetics. The D120C, D120N, and D120S mutants were shown to bind 1.6 ± 0.2, 1.8 ± 0.2, and 1.1 ± 0.2 mol of Zn(II) per monomer, respectively. The mutants exhibited 10- to 1000-fold drops in kcat values as compared with wild-type L1, and a general trend of activity, wild-type > D120N > D120C and D120S, was observed for all substrates tested. Solvent isotope and pH dependence studies indicate one or more protons in flight, with pKav values outside the range of pH 5–10 (except D120N), during a rate-limiting step for all the enzymes. These data demonstrate that Asp-120 is crucial for L1 to bind its full complement of Zn(II) and subsequently for proper substrate binding to the enzyme. This work also confirms that Asp-120 plays a significant role in catalysis, presumed via hydrogen bonding with water, assisting in formation of the bridging hydroxide/water, and a rate-limiting proton transfer in the hydrolysis reaction.

The ability of bacteria to acquire resistance to antibiotics is a serious problem that continues to challenge modern society (1). Excessive use and often misuse of antibiotics in the clinic and for agricultural purposes has resulted in tremendous selective pressure for antibiotic-resistant bacteria (2). These bacteria utilize a variety of methods to become resistant, including modification of cell wall components to prevent antibiotic binding, production of efflux pumps that transport the antibiotic out of the cell, and the production of enzymes that hydrolyze and render the antibiotic ineffective (1, 2).

The most common and least expensive effective antibiotics currently used are the β-lactams, such as carbapenems, cepha-

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likely due to the high activity of the enzymes, even in the crystalline state, toward all β-lactam-containing antibiotics (37, 38). Therefore, computational studies have been used extensively to study substrate binding, the role of the Zn(II) ions in catalysis, the protonation state of the active site, and inhibitor binding (37, 39–44). All of these models have made assumptions before the substrate was docked into the active site (37, 43), and some of these assumptions have been shown to be invalid for certain substrates (45). With L1, three key assumptions were made: 1) the bridging hydroxide functions as the nucleophile during catalysis, 2) Zn1 coordinates the β-lactam carbonyl, and 3) Zn2 coordinates the amide nitrogen of the β-lactam ring (37).

One of the residues identified through computational studies to be catalytically important in L1 is the aspartic acid at position 120 (the standard numbering scheme for class B β-lactamases is utilized herein (20)). From the crystal structure, Asp-120 clearly coordinates Zn2, with its unbound oxygen located directly under the bridging group in the active site (37). This is a geometry shared by many dinuclear metal-containing hydrolytic enzymes, including other metallo-β-lactamases and dioxygenases (46). Therefore, we believe that the findings of this work are applicable beyond L1 from S. maltophilia. In addition to its role as a metal-binding ligand, it has been hypothesized that Asp-120 electrostatically interacts with the bridging hydroxide, properly orienting it for nucleophilic attack on the substrate (37). This work describes our efforts to test this prediction and further our understanding the role of Asp-120 in both metal binding and substrate turnover. To probe the importance of this residue, three mutant enzymes were generated. Asp-120 was changed to a cysteine, an asparagine, and a serine to create D120C, D120N, and D120S, respectively (Fig. 1). Cysteine was substituted to allow for continued binding of Zn2 but eliminate any interaction of the residue with the bridging hydroxide/water. Asparagine was chosen as a chemically different but structurally similar surrogate for aspartic acid, allowing for continued binding of Zn2 and providing a moiety for interaction with the bridging hydroxide/water. Replacement of aspartic acid with serine was intended to remove both the metal-binding ability of the residue at this position and any ability to interact with the bridging hydroxide/water.

EXPERIMENTAL PROCEDURES

Escherichia coli strains DH5α and BL21(DE3) were obtained from Invitrogen and Novagen, respectively. Plasmids pET26b and pUC19 were purchased from Novagen. Primers for sequencing and mutagenesis studies were purchased from Integrated DNA Technologies. Deoxynucleotide triphosphates (dNTPs), MgSO4, Thermopol buffer, Deep Vent DNA polymerase, and restriction enzymes were purchased from Promega or New England Biolabs. Polymerase chain reaction was conducted using a Thermolyne Ampliclon II unit. DNA was purified using the Qiagen QIAquick gel extraction kit or Plasmid Purification kit with Qiagen QIAquick gel extraction kit or Plasmid Purification kit. Asp-120 was changed to a cysteine, an asparagine, and a serine to create D120C, D120N, and D120S, respectively (Fig. 1).

FIG. 1. Pictorial representation of the active site of wild-type L1 rendered using Chem Draw Ultra v. 5.0 (top) and Rasmol 2.6 (bottom). Drawing indicates the proposed interaction of Asp-120 with the bridging hydroxide and changes affected by each of the L1 mutants. The coordinates for the Rasmol figure were obtained from the Protein Data Bank using the accession number 1sm1.

Asp-120 Mutants of Metallo-β-lactamase L1

Asp-120 Mutants of Metallo-β-lactamase L1 rendered using Chem Draw Ultra v. 5.0 (top) and Rasmol 2.6 (bottom). Drawing indicates the proposed interaction of Asp-120 with the bridging hydroxide and changes affected by each of the L1 mutants. The coordinates for the Rasmol figure were obtained from the Protein Data Bank using the accession number 1sm1.
tometer with atomic emission spectroscopy detection (ICP-AES) was used to determine the metal content of multiple preparations of wild-type L1 and L1 mutants. Calibration curves were based on four standards and had correlation coefficient limits of at least 0.9950. The final dialysis buffer was used as a blank. The emission line of 213.856 nm is the most intense for zinc and was used to determine the zinc content in the samples. The errors in metal content data reflect the standard deviation (σ), of multiple enzyme preparations. A second analysis of metal content was preformed on enzyme samples that were incubated for 1 h, on ice, in buffer containing a final concentration of 100 μM ZnCl2. These “metal-saturated” samples were then dialyzed versus 2× 1-liter metal-free buffer for a total of 4 h, and metal content was analyzed by ICP-AES as described above.

Circular dichroism samples were prepared by dialyzing the purified enzyme samples versus 3× 2 liters of 5 mM phosphate buffer, pH 7.0, over 6 h. The samples were diluted with final dialysis buffer to ~75 μg/ml. A JASCO J-810 CD spectropolarimeter operating at 25 °C was used to collect CD spectra.

Assays were conducted at 25 °C in 50 mM cacodylate buffer, pH 7.0, containing 100 μM ZnCl2 on an HP 5480A diode array UV-visible spectrophotometer. The changes in molar absorptivities (Δε) used to quantitate products were (in M−1 cm−1): nitrocefin, Δε485 = 17,420; cephalothin, Δε485 = −8,790; cefoxitin, Δε485 = −7,000; cefadroxil, Δε485 = −6,410; imipenem, Δε390 = −9,000; meropenem, Δε390 = −7,600; biapenem, Δε485 = 8,600; ampicillin, Δε485 = −809; and penicillin G, Δε390 = −989. When possible, substrate concentrations were varied between 0.1 and 10 times the Kcat, and changes in absorbance (ΔA) versus time data were measured for a period of 60 s for each substrate concentration. In kinetic studies using substrates with low Kcat values (cefotaxim, nitrocefin, and cephalothin) or with small Δε values (penicillin and ampicillin), substrate concentrations were varied between 0.1 and 10 times Kcat and as many as 16 different points of the ΔA versus time data as possible was used to determine the velocity. Steady-state kinetic constants, Kcat, and Kcat, were determined by fitting initial velocity versus substrate concentration data directly to the Michaelis equation using Igor Pro (36). The reported errors reflect fitting uncertainties. All steady-state kinetic studies were performed in triplicate with recombinant L1 from at least three different enzyme preparations.

pH dependence studies were performed as described above but using a buffer system containing 25 mM MES, 50 mM Tris, 25 mM CHES, 10 mM NaCl, and 100 μM ZnCl2 (MTCN). Buffers for each pH tested were made from a common 10X stock of MTCN buffer. The pH of each was then adjusted to the desired value using either 6 M HCl or 10 M NaOH, and the appropriate volume of aqueous ZnCl2 was added to reach a concentration of 100 μM. Kcat, and Kcat, were determined as described above, and log plots of those values versus pH were generated using Igor Pro.

Steady-state kinetic assays were conducted at 25 °C in 50 mM cacodylate buffer, pH 7.0, containing 100 μM ZnCl2 and a range in D2O concentrations from 0 to 100%. On a HP 5480A diode array UV-visible spectrophotometer. Steady-state kinetic constants, Kcat, and Kcat, were determined by fitting initial velocity versus substrate concentration data directly to the Michaelis equation using Igor Pro (36). Plots of kcat versus % D2O were generated using Igor Pro. The reported errors reflect fitting uncertainties.

Rapid-scanning visible spectra of nitrocefin hydrolysis by L1, and the L1 mutants were collected on a Applied Photophysics SX.18MV stopped-flow spectrophotometer equipped with an Applied Photophysics PD.1 photodiode array detector and a 2-mm path length optical cell. The wild-type L1 experiment consisted of 25 μM enzyme and 5 μM nitrocefin in a cacodylate buffer containing 100 μM ZnCl2. During the reaction temperature was thermostated at 25 °C, and the spectra were collected between 300 and 725 nm. Data from at least three experiments were collected and averaged. Absorbance data were converted to concentration data as described previously by McMannus and Crowder (51). Due to weaker binding and slower turnover of substrate with the L1 mutants, enzyme concentrations of 50 μM were used with 5 μM nitrocefin utilizing the same buffer system and experimental conditions as in the wild-type L1 experiment. Stopped-flow fluorescence studies of nitrocefin hydrolysis by L1 were performed on an Applied Photophysics SX.18MV spectrophotometer, using an excitation wave-length of 295 nm and a WG320 nm cut-off filter on the photomultiplier. These experiments were conducted at 10 °C using the same buffer as in the rapid-scanning visible studies. Fluorescence data were fitted to kcat = (d[S]/d[⋅]) + k0 as described previously (52) or to kcat = k[S] + k0 by using CurveFit version 1.0.

**RESULTS**

Wild-type L1, D120C, D120N, and D120S were overexpressed in *E. coli* and purified as previously described (36). This procedure produced an average of 50–60 mg of >95% pure, active protein per 4 liters of growth culture. Circular dichroism spectra were collected on samples of wild-type, and each of the mutants to ensure the proteins produced using the pET26b overexpression system had the correct secondary structure. The CD spectra (data not shown) of wild-type L1 and the mutants were identical. Metal analyses on multiple preparations of wild-type L1 demonstrated that the enzyme binds 1.9 ± 0.2 Zn(II) ions per monomer (Table I), in agreement with previous results (36). Metal analysis on multiple preparations of D120C, D120N, and D120S showed 1.6 ± 0.2, 1.8 ± 0.2, and 1.1 ± 0.2 Zn(II) ions per monomer, respectively.

**Table I**

| Enzyme | Kcat with nitrocefin | Solvent iso | Kd (μM) |
|--------|----------------------|------------|---------|
| Wt     | 1.9 ± 0.3            | 0.2        | 2.0 ± 0.03 |
| D120C  | 1.6 ± 0.2            | 0.2        | 1.47 ± 0.05 |
| D120N  | 1.8 ± 0.2            | 0.2        | 5.36 ± 0.22 |
| D120S  | 1.1 ± 0.2            | 0.2        | 1.87 ± 0.06 |

The errors in metal content data reflect the standard deviation of at least five determinations. The metal analyses on multiple preparations of wild-type L1 were identical. Metal analyses on multiple preparations of wild-type L1 and each of the mutants with nine substrates. These values are presented in Tables II–IV. When using nitrocefin as substrate and 50 mM cacodylate, pH 7.0, as the buffer, wild-type L1 exhibited a kcat value of 38 ± 1 s−1 and a Kcat value of 12 ± 1 μM. The inclusion of 100 μM ZnCl2 in the assay buffer resulted in slightly lower values of Kcat and higher values for kcat (36). The inclusion of higher concentrations of Zn(II) did not further affect the steady-state kinetic constants. Four cephalosporins (cefalexin, cefoxitin, cephalothin, and nitrocefin), three carbapenems (biapenem, imipenem, and meropenem), and two penicillins (penicillin G and ampicillin) were utilized as representatives of the three major classes of β-lactam containing antibiotics. The preference of L1 for penicillins and carbapenems over cephalosporins, as exemplified by the kcat values, is in agreement with previous studies and supports the placement of L1 in the β-lactamase Bc family (9). For all substrates tested, the mutant enzymes exhibited reduced kcat values as compared with wild-type L1, with D120N being the most active of the three. A general trend of activity was observed for all substrates tested: wild-type L1 > D120N > D120C and D120S (Tables II–IV). There does not seem to be an observable trend with respect to the activities of D120C and D120S. In most cases they exhibited kcat values very similar to one another. D120N exhibited an activity fourteen to thirty-seven times lower than wild-type L1 toward carbapenems. However, this was generally ten or more times greater than the activity of either D120C or D120S with the same substrates. With the exception of cefoxitin, D120N exhibited kcat values seven to sixteen times lower than wild-type L1, but fourteen (or more) times greater than that of D120C or D120S toward cephalosporins. Although the overall trend with kcat values holds true for cefoxitin, it would appear that none of the enzymes, including wild-type L1, hydrolyzed this compound well. On average, D120N showed ten times less activity toward penicillins than

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1 The abbreviations used are: ICP-AES, inductively coupled plasma-emission spectroscopy; L1, metallo-β-lactamase from *S. maltophilia*; MTCN, MES-TRIS-CHES-NaCl buffer; CD, circular dichroism; MES, 4-morpholinoethanesulfonic acid; CHES, 2-cyclohexylamino/ethanesulfonic acid.
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**Steady-state kinetics constants for wild-type L1 and L1 mutants with carbapenems**

| Biapenem          | Imipenem          | Meropenem         |
|-------------------|-------------------|-------------------|
| $k_{cat}$         | $K_m$             | $k_{cat}/K_m$     | $k_{cat}$         | $K_m$             | $k_{cat}/K_m$     |
| $s^{-1}$          | $\mu M$           | $s^{-1}$          | $\mu M$           | $s^{-1}$          | $\mu M$           |
| Wt 134 ± 4        | 32 ± 1            | 4.2              | 370 ± 5           | 57 ± 7            | 6.5              |
| D120C 0.30 ± 0.02 | 45 ± 7            | 0.0067           | 0.36 ± 0.02       | 65 ± 7            | 0.0055           |
| D120N 3.6 ± 0.4   | 128 ± 2           | 0.028            | 15 ± 2            | 310 ± 64          | 0.048            |
| D120S 0.50 ± 0.01 | 81 ± 4            | 0.0062           | 0.63 ± 0.02       | 94 ± 6            | 0.0067           |
| D120N           | 15 ± 2            | 310 ± 64         | 0.048            | 94 ± 6            | 0.0067           |

**Steady-state kinetics constants for wild-type L1 and L1 mutants with cephalosporins**

| Cefaclor          | Cefoxitin         | Cephalothin       | Nitrocefin        |
|-------------------|-------------------|-------------------|-------------------|
| $k_{cat}$         | $K_m$             | $k_{cat}/K_m$     | $k_{cat}$         | $K_m$             | $k_{cat}/K_m$     |
| $s^{-1}$          | $\mu M$           | $s^{-1}$          | $\mu M$           | $s^{-1}$          | $\mu M$           |
| Wt 42 ± 1         | 13 ± 1            | 3.2              | 1.9 ± 0.1         | 1.1 ± 0.1         | 1.7              |
| D120C 0.30 ± 0.02 | 98 ± 15           | 0.0331           | 0.56 ± 0.04       | 55 ± 8            | 0.010            |
| D120N 6.3 ± 0.6   | 550 ± 76          | 0.011            | 1.53 ± 0.07       | 98 ± 9            | 0.016            |
| D120S 0.28 ± 0.01 | 80 ± 8            | 0.0035           | 0.34 ± 0.01       | 53 ± 5            | 0.0064           |
| D120S           | 0.28 ± 0.01       | 80 ± 8           | 0.0035           | 0.34 ± 0.01       | 53 ± 5           |

**Steady-state kinetics constants for wild-type L1 and L1 mutants with penicillins**

| Ampicillin        | Penicillin G      |
|-------------------|-------------------|
| $k_{cat}$         | $K_m$             | $k_{cat}/K_m$     | $k_{cat}$         | $K_m$             | $k_{cat}/K_m$     |
| $s^{-1}$          | $\mu M$           | $s^{-1}$          | $\mu M$           | $s^{-1}$          | $\mu M$           |
| Wt 520 ± 10       | 55 ± 5            | 9.5              | 600 ± 100         | 38 ± 2            |
| D120C <0.01       | NA*               | NA               | <0.01             | NA                |
| D120N 77 ± 10     | 1332 ± 257        | 0.058            | 45 ± 2            | 384 ± 40          |
| D120S <0.01       | NA                | NA               | <0.01             | NA                |

* NA, no activity.

wild-type L1. Interestingly hydrolysis of penicillins was virtually undetectable with both D120C and D120S. Because $K_m$ values are often used as a first approximation of substrate binding, the $K_m$ values exhibited by the mutants were compared with those of wild-type L1. All three mutants exhibited larger $K_m$ values than wild-type L1. Typically with the substrates tested, D120N had the largest $K_m$ values; however, a clear trend for $K_m$ values was not apparent.

To probe further the binding of nitrocefin to wild-type L1 and the mutants, stopped-flow fluorescence studies were conducted as previously described (52). The reaction of enzyme with nitrocefin under steady-state conditions at 10 °C resulted in a rapid decrease in fluorescence followed by a rate-limiting return of fluorescence (data not shown). Fitting of the data, as described by Spencer et al. (52), yielded $K_m$ values for wild-type L1 and the three mutants (Table I).

Rapid-scanning visible spectra of 25 μM wild-type L1 with 5 μM nitrocefin demonstrated a decrease in absorbance at 390 nm, an increase at 485 nm, and a rapid increase and slower decrease in absorbance at 665 nm. These spectra are similar to those previously reported for wild-type L1 and nitrocefin (51), and the features can be attributed to substrate decay, product formation, and formation and decay of a ring-opened, nitrogen anionic intermediate, respectively (51, 53, 54). Under these conditions, the −0.7 μM intermediate was formed during the first 10 ms of the reaction (Fig. 2), and the rate of decay of this intermediate corresponds to the steady-state $k_{cat}$ (Tables II–IV). Extending the time scale of scanning and using 50 μM enzyme, due to weaker substrate binding by the mutants, and 5 μM nitrocefin, rapid-scanning visible spectra of D120C, D120N, and D120S showed similar changes in absorbance at 390 nm and 485 nm; however, only D120N gave rise to any change in absorbance at 665 nm. This absorbance change was significantly less than wild-type and corresponds to only −0.1 μM intermediate formation.

**DISCUSSION**

Mutations were introduced into L1 using the overlap extension method (47), and three indicators were analyzed to probe whether the single point mutations resulted in large structural changes in these mutants. Overexpression levels, total amounts of isolatable enzyme after protein purification, and CD spectra of the mutants were compared with those of wild-type L1. All mutants exhibited results virtually identical to those of wild-type L1 for all three indicators, leading to the conclusion that none of the point mutations resulted in large structural changes in L1 and that any kinetic and metal binding differences could be attributed to the changed amino acid.

In enzymes, Asp residues can have three major functions: 1) coordination of metal ions, 2) hydrogen bonding with active site residues and substrates, and 3) shuttling protons to and from groups in the active site. Our data and previous studies (37) clearly show that one of the Asp-120 oxygens is involved in coordination of metal ions, 2) hydrogen bonding with active site residues and substrates, and 3) shuttling protons to and from groups in the active site. Our data and previous studies (37) clearly show that one of the Asp-120 oxygens is involved in coordination of metal ions, hydrogen bonding with active site residues and substrates, and shuttling protons to and from groups in the active site.
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Fig. 2. Formation of ring-opened, nitrogen anion intermediate by wild-type L1 and L1 mutants. Spectra were collected using rapid scanning visible studies, and the absorbance values at 668 nm were converted to concentration values as described under "Experimental Procedures." Typical reactions were conducted with 25 μM L1 (50 μM mutants due to reduced activity) and 5 μM nitrocefin in 50 mM cacodylate, pH 7.0, containing 100 μM ZnCl₂ at 25 °C.

D120C and D120N bound Zn(II) at levels comparable to wild-type L1, 1.6 ± 0.2, 1.8 ± 0.2, and 1.9 ± 0.2 mol of Zn(II) per monomer, respectively (Table I). D120S only bound 1.1 ± 0.2 mol of Zn(II) per monomer, as was expected with the removal of a metal-binding ligand at the Zn₂ site in this mutant.

From previous work it was known that recombinant wild-type L1 exhibited its lowest Km values and greatest kcat values with 100 μM ZnCl₂ in the assay buffer (36). Similarly, the mutants exhibited their lowest Km values and greatest kcat values with 100 μM ZnCl₂ in the buffer. This indicated that the enzymes as isolated do not bind their full complement of Zn(II) but can incorporate available Zn(II) into their active sites. Confirming this notion, ICP-AES analysis of wild-type L1, D120C, and D120N that had been incubated in buffer containing 100 μM ZnCl₂ revealed increased levels of Zn(II) bound to the enzymes (no increase in the amount of bound Zn(II) was observed with D120S). Subsequently, to ensure saturation of the metal-binding sites and to facilitate direct comparison of the kinetic data, 100 μM ZnCl₂ was included in all of the kinetic buffers used.

To compare the activity of the mutants with wild-type L1, we examined the steady-state kinetics of three carbapenems, four cephalosporins, and two penicillins with each enzyme. The substrates tested were chosen, because they exhibited low Km values in previous kinetic studies (36), and we believed that we could saturate the enzymes with substrate even if there was large change in binding with the point mutations. It is not surprising that the smallest kcat values were obtained with cephalosporins, affirming the classification of L1 as a group Bc metallo-β-lactamase (preference toward penicillins and carbapenems) (9). In particular, it would appear that L1 has little preference for cefoxitin, exhibited by a kcat/Km value of 1.7 s⁻¹ μM⁻¹. For all other substrates the mutants showed significantly altered activities, with decreases in kcat values on the

2 A. Carenbauer, unpublished data.
order of 10- to 1000-fold. The greatest reduction from wild-type L1 is seen with D120C and D120S, with decreases ranging from 100- to 1000-fold in $k_{cat}$. The difference between D120N and wild-type L1 is less exaggerated with reductions in $k_{cat}$ values ranging from 7- to 37-fold. In most cases, except for cefoxitin, D120N exhibited a 10- to 20-fold greater activity than either D120C or D120S.

We first address the reduction in activity observed with D120S. Our data clearly indicate that D120S is a mononuclear enzyme, even under metal-saturating conditions (Table I). This is not surprising, because there is no evidence to suggest that the pK_a of the serine residue would be lowered sufficiently to allow for the deprotonation of the hydroxyl and permit it to coordinate Zn(II). Repeated attempts to determine a $K_a$ for D120S with nitrocefin, via stopped-flow fluorescence, failed due to a lack of any observable consistent change in fluorescence. Under a wide range of enzyme and nitrocefin concentrations, no consistent detectable change in fluorescence could be observed (data not shown). In contrast to D120S, metal analysis of wild-type L1, D120C, and D120N indicates that all are dinuclear enzymes and not surprisingly had $K_a$ values with nitrocefin fairly similar to one another, 37 ± 6 μM, 38 ± 7 μM, and 97 ± 14 μM, respectively. It is logical to conclude then that the reduced catalytic activity of D120S is due to its inability to bind substrate. This confirms the proposition of Ullah et al. (37) that in L1, both Zn(II)s are necessary for tight substrate binding and in turn full catalytic activity of the enzyme. It should be noted that this result is contrary to recently published work (59) asserting that β-lactamases, such as L1 and CcrA (metallo-β-lactamase from B. fragilis), are apo- (without metal) or mononuclear enzymes in vivo.

The kinetic data for the D120C mutant indicate, however, that the presence of both Zn(II)s alone and, subsequently, the ability to bind substrate, do not render an active enzyme. This suggests a role of the unbound oxygen of Asp-120. For all substrates tested, the exception being cefoxitin as previously noted, there is a clear trend observed in the steady-state kinetic data: wild-type L1 > D120N > D120C and D120S. The similarity in the kinetic data for D120C and D120S can be attributed to residual hydrolysis of substrate due to template and scaffolding effects (60). The possibility of purely background hydrolysis due to the excess Zn(II) in the buffer was ruled out, because reactions of just substrate and buffer show <<1% hydrolysis during normal reaction times (data not shown). Further evidence supporting this claim is seen in the rapid scans of nitrocefin hydrolysis by wild-type L1 and the mutants. Both D120C and D120S show minimal to no accumulation of intermediate as seen with wild-type L1 and D120N (Fig. 2), suggesting that a mechanism other than that of wild-type L1 is occurring with these enzymes. This is consistent with hydrolysis due to a template effect (60).

What then is the role of the unbound oxygen of Asp-120? At physiological pH, it is highly improbable that an Asp bound to a Zn(II) is protonated and can take part in a direct proton transfer. Our data along with previous information (37, 51) support the idea that the unbound oxygen of Asp-120 is interacting, via hydrogen bonding, with the group bridging the two Zn(II) ions. It is expected that at neutral pH levels, the bridging group is a hydroxide. Upon substrate binding, we predict that the bridging hydroxide becomes terminally bound to Zn_2 while retaining a hydrogen bond to Asp-120. As predicted with model complexes (61), other Zn(II)- and Fe-containing proteins (62-65), computational studies on metallo-β-lactamases (40, 42, 66), and with other metallo-β-lactamases (57, 67, 68), this metal bound hydroxide then serves as the nucleophile that attacks the β-lactam carbonyl. The kinetic data clearly show
that D120N is the only mutant that retains significant hydrolytic activity. Not surprisingly, because D120N is the only mutant that retains both of the apparently critical requirements for substrate turnover we investigated, namely the effective binding of substrate and a residue at position 120 capable of electrostatically interacting with the putative nucleophile. The reduced $k_{cat}$ values observed with D120N are likely due to the chemical difference of the moiety interacting with the nucleophile. It would appear that in this mutant the Asn oxygen is coordinating Zn$_2$ (69, 70), leaving the lone pair of the amine to interact with the nucleophile. This is supported by the pH dependence and proton inventory data for D120N. With wild-type L1, there are no observable $pK_a$ values between pH 5 and 10 (Fig. 3), and the proton inventory with nitrogen indicates a single proton in flight during a rate-limiting step of the reaction (Fig. 5). pH dependence plots of D120N with nitrogen, however, indicate the possibility of multiple $pK_a$ values between pH 9.5 and 10 (Fig. 4), and proton inventories show multiple protons in flight during a rate-limiting step of the reaction.

Why is this electrostatic interaction between the putative nucleophile and residue 120 catalytically important? Currently, there are two proposed reaction mechanisms for the dinuclear Zn(II) $\beta$-lactamases, depending on the substrate. When nitrogen is used as the substrate, a ring-opened, nitrogen anionic intermediate is thought to form, and the protonation of this nitrogen anion is rate-limiting (51, 54, 68). When nitrocefin is used as the substrate, a ring-opened, nitro-$\beta$-lactam intermediate, which occurs via the protonation of the nitrogen anion, is thought to be rate-limiting (52). The protonation of this nitrogen anion is rate-limiting (51, 54, 68). When nitrocefin is used as the substrate, a ring-opened, nitro-$\beta$-lactam intermediate, which occurs via the protonation of the nitrogen anion, is thought to be rate-limiting (52). The protonation of this nitrogen anion is rate-limiting (51, 54, 68). When nitrocefin is used as the substrate, a ring-opened, nitro-$\beta$-lactam intermediate, which occurs via the protonation of the nitrogen anion, is thought to be rate-limiting (52). The protonation of this nitrogen anion is rate-limiting (51, 54, 68).

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Metal Binding Asp-120 in Metallo-β-lactamase L1 from *Stenotrophomonas maltophilia* Plays a Crucial Role in Catalysis
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