Exploring the Role and the Binding Affinity of a Second Zinc Equivalent in *B. cereus* Metallo-β-lactamase[†]

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ABSTRACT: Metallo-β-lactamases are a newly characterized family of zinc enzymes present in several pathogenic strains that represent an emerging clinical threat. Enzymes from different organisms exhibit an outstanding functional diversity, particularly in the metal ion requirements for activity. We have investigated the effect of the second zinc(II) equivalent in the enzyme βLII from *Bacillus cereus*, naturally active in the mono-zinc(II) form. The enzyme is reversibly inactivated at low pH, due to dissociation of the two zinc(II) equivalents. The pH profile indicates that zinc-bound water in the mono-zinc(II) enzyme possesses a pK_a below 4.9, indicating that a second zinc(II) equivalent is not needed for nucleophile activation. Instead, the second zinc(II) may contribute to properly anchor Asp120, that ultimately orients the attacking nucleophile in binuclear enzymes. This role may be fulfilled by Arg121 in mono-zinc enzymes, as suggested by the kinetic study of the R121C mutant in βLII. In addition, it is demonstrated that Arg121 is not responsible for the low binding affinity of βLII toward a second zinc(II) equivalent.

The most prevalent resistance mechanism developed by bacteria to escape the action of β-lactam antibiotics consists of the production of β-lactamases (EC 3.5.2.6), i.e., enzymes capable of hydrolyzing the cyclic amide bond of these compounds (*1, 2*). These enzymes have been classified into four classes (A, B, C, and D) according to sequence homology (*3*−*5*). Generically, they can be grouped into serine-active (classes A, C, and D) and metallo-β-lactamases (class B). The first group of enzymes uses an active site serine residue as a nucleophile, while the activity of class B enzymes depends on the presence of one or two zinc(II) ions in their active site. An immediate spread of metallo-β-lactamases to pathogenic bacteria has raised the concern of the biomedical community (*6, 7*).

All hitherto characterized metallo-β-lactamases exhibit a similar fold and highly conserved active sites (*8*−*10*). Notwithstanding these similarities, they display a remarkable molecular diversity which is thought to be responsible for fine-tuning the catalytic properties of enzymes from different bacterial strains. Metallo-β-lactamases can bind up to two zinc(II) ions in their active sites (*8*−*10*). Mononuclear and binuclear sites have been characterized, the enzyme activity being dependent on the metal content (*11*−*17*). It is now becoming clear that enzymes from different bacterial strains exhibit distinctive metal binding affinities, and each zinc-(II) site may play a specific role in the enzyme activity.

The enzymes CcrA[1] from *Bacteroides fragilis* (*18*), IMP-1 from *Pseudomonas aeruginosa* (*19*), and L1 from *Stenotrophomonas maltophilia* (*20*) bind tightly two zinc(II) equivalents, which seem to be essential for catalysis (*17, 21*−*23*). In contrast, βLII from *Bacillus cereus* is able to bind up to two zinc(II) ions, but with quite different affinities (*11, 12, 15, 16*). The crystal structures of both binuclear and mononuclear forms of βLII have been solved (Figure 1) (*13, 24, 25*). βLII is most probably active in vivo as a mono-zinc(II) enzyme. The fact that the incorporation of a second zinc(II) equivalent enhances the activity of the mono-zinc-(II) species has led Fabiane et al. to suggest that tightening the binding of the second zinc(II) could be an evolutionary advantage for the more efficient enzymes from pathogenic bacteria (*24*).

The high resemblance of the binuclear active sites of CcrA and βLII does not correlate with the distinct binding affinities of these enzymes toward bivalent cations. A detailed comparison of the metal site environment reveals that an Arg residue is located close to the second zinc(II) in βLII (*18, 24*). This residue is not strictly conserved in all lactamases. Arg121 [standard BBL numbering (*26*)] is replaced by a Cys in CcrA (*27*), and by a His residue in L1, that is able to bind the metal ion (*20, 28*). It has been suggested that mutation of the Arg by a neutral residue would enhance the zinc(II) binding capabilities of these enzymes (*18, 24, 25*).

Different catalytic mechanisms have been proposed for the mono- and binuclear enzymes (*29, 30*). In the first case, the zinc-bound water/hydroxide is expected to be the attacking nucleophile (*29*). In binuclear enzymes, this water/

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[1] Abbreviations: βLII, *Bacillus cereus* metallo-β-lactamase; CcrA, *Bacteroides fragilis* metallo-β-lactamase; L1, *Stenotrophomonas maltophilia* metallo-β-lactamase; IMP-1, *Serratia marcescens* metallo-β-lactamase; EDTA, ethylenedinitrilotetraacetic acid; BSA, bovine serum albumin.

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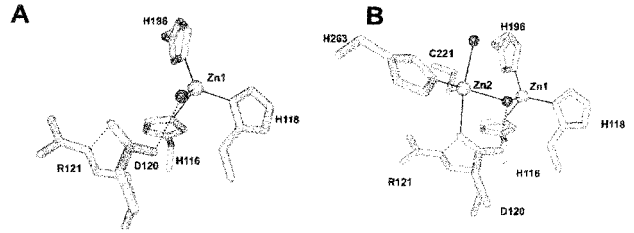

FIGURE 1: Schematic representation of the *B. cereus* βLII active site in the (A) mono-zinc(II) [pdb code 3bc2] and (B) bi-zinc(II) forms [pdb code 1bc2 (*24*)]. Dark spheres represent water/OH molecules. Residues are labeled according to the BBL standard numbering (*26*). This figure was drawn using Molmol (*69*).

hydroxide bridges the two zinc(II) ions (*18*−*20*). A hydroxide group bridging the two zinc(II) ions should certainly exhibit a lower pK_a compared to the mono-zinc derivative. A pK_a of 5.6−5.8 has been attributed to the protonation of the zinc-bound water in mono-zinc(II) βLII (*29*). In contrast, no titratable group affecting the hydrolytic activity between pH values of 5.25 and 10 has been noticed in the binuclear enzyme of *B. fragilis* (*31*). The presumed lower pK_a value in the binuclear enzyme has been thus attributed to the simultaneous binding of the water molecule to two zinc(II) ions, even if this coordination mode may impair the nucleophilicity of the attacking hydroxide.

Understanding the possible evolutionary advantages of a second zinc(II) ion is a major issue in the field of metallo-β-lactamases. This involves finding a rationale for the existence of mono- and bi-zinc enzymes, as well as uncovering the structural features that tune metal binding. The different affinity of the two zinc equivalents in βLII allows us to study separately the mono- and bi-zinc(II) derivatives (*15*, *16*). In this work, we would like to address the following issues: (1) Is the binding of a second zinc(II) ion necessary for lowering the water pK_a in metallo-β-lactamases? (2) Can the binding affinity toward the second zinc(II) equivalent be improved by replacement of Arg121 in βLII?

EXPERIMENTAL PROCEDURES

Materials. Chemicals were of the best quality available. Metal-free buffers were prepared by adding Chelex 100 to normal buffers and stirring for half an hour. Restriction endonucleases and other DNA modifying enzymes were purchased from Promega. Oligonucleotides were synthesized by Biosynthesis, Inc. (Lewisville, TX). Benzylpenicillin and cephaloridine were purchased from Sigma. Nitrocefin was a kind gift of Glaxo Wellcome. Atomic absorption standard solutions of cobalt(II) and zinc(II) salts were used for titrations and preparation of metal derivatives.

Site-Directed Mutagenesis and Enzyme Purification. The mutation on Arg121 was performed by oligonucleotide-directed mutagenesis on single-stranded DNA (*32*). *E. coli* CJ236 cells harboring the plasmid pKS-NH_3^+, which contains a fragment coding for the first 112 amino acids of βLII, were infected with the helper phage R408. Single-strand plasmid DNA was purified from phage particles. Double-strand synthesis was carried out using the Klenow fragment of DNA polymerase I from *E. coli*, primed using the following DNA oligonucleotide: 3′-GTGTACGCGT**A**CGACTA**A**C**A**TAAC-CGCC-5′. This sequence contains the desired R121C mutation and a translationally silent mutation introducing a *Sph*I restriction site (underlined). The in vitro synthesized double-strand DNA was transformed into *E. coli* JM109 cells, and clones containing the *Sph*I restriction site were selected and sequenced. The whole βLII gene was reconstructed by ligation of the mutated amino-terminal fragment with the wild-type carboxy-terminal fragment into the expression vector pETGEX-CT (*33*). Both wild-type and mutant proteins were expressed as fusion proteins with glutathione-*S*-transferase, and purified as previously described (*15*). The purity of all expressed proteins was confirmed by SDS−PAGE. The concentration of the purified enzymes was determined by measuring the absorbance at 280 nm of enzyme preparations using an extinction coefficient of 30 500 M^{-1} cm^{-1}(*16*).

Apoenzyme Preparation, Metal Substitution, and UV−*Vis Spectroscopy.* The wild-type and mutant apoenzymes were prepared by adding EDTA until a final concentration of 5 mM to 1−2 mg/mL enzyme solutions, and incubating for 5 min at room temperature. EDTA was removed by three dialysis steps of 2−3 h each, against 100 volumes of metal-free sodium succinate 20 mM at pH 6.0, NaCl 1 M. The apoenzymes retained <1% the activity of the holoenzymes, and full activity was recovered upon addition of zinc(II). The cobalt(II) adducts were generated by adding excess $CoCl_2$ to the apoenzyme solutions. The electronic spectra of the cobalt(II) adducts were recorded in a Gilford Response II spectrometer.

Metal Binding. Metal binding during turnover was followed by measuring the hydrolysis rates of 1 mM benzylpenicillin in the presence of increasing zinc(II) concentrations (*15*). The substrate and the apoenzyme were mixed in 20 mM metal-free TACS buffer (Tris 50 mM, sodium cacodylate 50 mM, sodium acetate 50 mM, NaCl 500 mM) at pH 6.0, and then zinc(II) was added to achieve the desired final concentration. Under these experimental conditions, the spontaneous hydrolysis of benzylpenicillin was negligible. The quartz cuvettes were washed with 20% HNO_3, 0.1 M NaOH and finally with water, after each measurement to remove traces of zinc(II). The data were fit to the following equation:

$$rate = \frac{a}{\frac{[Zn]}{K_{D2}} + 1 + \frac{K_{D1}}{[Zn]}} + \frac{b}{1 + \frac{K_{D2}}{[Zn]} + \frac{K_{D1} \cdot K_{D2}}{[Zn]}} \quad (1)$$

where *a* and *b* are the hydrolysis rates for the mononuclear and binuclear enzymes, respectively, and K_{D1} and K_{D2} are the apparent dissociation constants for the two zinc(II) equivalents in the presence of the substrate. This approach assumes that the apoenzyme is completely inactive, the mono-zinc(II) enzyme is partially active, and the bi-zinc(II) enzyme is fully active. The data were fit by normalizing the rates to 100% for the plateau (i.e., *a* was fixed equal to 100) whereas *b* and the apparent dissociation constants were obtained by nonlinear fit of the data points. We assumed that the free zinc(II) concentration was equal to total zinc-(II) concentration as the metal was always in a large excess with respect to the enzyme concentration (typically of 70 nM).

The metal content of the enzyme in the resting state at pH 5.0 and 6.0 and at different added zinc(II) concentrations was measured by atomic absorption spectroscopy in a

Metrolab 250 AA atomic absorption spectrometer operating in the flame mode. Typically, 1 mL samples of the enzyme at a concentration of ca. 50 μM were dialyzed against 150 mL of TACS buffer (four changes) over 24 h with different zinc(II) concentrations [0, 0.2, 20, and 100 μM zinc(II)]. The dialysis membranes, buffers, and plasticware were rigorously treated by following standard procedures (*34*, *35*). Protein concentration was quantified by UV−visible spectroscopy. The zinc(II) content of the final dialyisis solution as results from atomic absorption measurements was subtracted in each case. All samples were fully active after the dialysis steps.

Kinetic Measurements and pH Dependence of Kinetic Parameters. Benzylpenicillin hydrolysis was followed by monitoring the changes in absorbance at 235 nm. Concentrations were calculated using $\Delta\epsilon_{235} = 800\ M^{-1}\cdot cm^{-1}$. The complete reaction time courses were recorded on an Ultraspec II LKB spectrometer using a 0.1 cm light path cuvette. The initial concentration of benzylpenicillin was 3 mM, and the enzyme concentrations ranged from 140 to 830 nM for the wild-type enzyme and from 280 to 1100 nM for the mutant enzyme. The reaction medium always contained 50 μg/mL bovine serum albumin. The kinetic parameters K_M and k_{cat} were estimated by nonlinear fitting of the data to the integrated form of the Michaelis−Menten equation. The sizable spontaneous hydrolysis of the substrate at pH values higher than 7 at relatively high zinc(II) concentrations did not allow us to subtract it adequately from the enzyme-catalyzed time courses. All measurements were performed at 25 °C.

The pH dependence of the kinetic parameters for benzylpenicillin hydrolysis was determined by performing measurements in the polybuffer TACS in the presence of different zinc(II) concentrations. The pH value was adjusted in the reaction cuvette by addition of aliquots of 5 M HCl, and the reaction was started afterward. The final pH in the cuvette was found to decrease less than 0.02 pH unit. The enzymes were stable in the reaction medium for at least 4 times the total assay time even at pH 4.5. The experimental data were fit to eqs 2 and 3, that assume a two-protonation event:

$$k_{cat} = \frac{k_{cat}^{max}}{1 + 10^{(pH - pK_{a1})} + 10^{(2\cdot pH - pK_{a1} - pK_{a2})}} \quad (2)$$

$$k_{cat} = \frac{k_{cat}^{max}}{1 + 10^{(2\cdot pH - 2\cdot pK_{a})}} \quad (3)$$

Equation 3, that assumes a simultaneous deprotonation process, describes better the experimental data (Figure 2). The pK_a values reported here result from this fit.

The catalytic efficiency of both wild-type and R121C βLII against other substrates was measured in TACS buffer at pH 6.0 in the presence of 20 μM zinc(II). Complete reaction time courses were recorded spectrophotometrically, and substrate concentrations were calculated using the following extinction coefficients and wavelength maxima: ampicillin, $\Delta\epsilon_{232} = 880\ M^{-1}\ cm^{-1}$; cefotaxime, $\Delta\epsilon_{263} = 4800\ M^{-1}\ cm^{-1}$; nitrocefin, $\Delta\epsilon_{485} = 17\,400\ M^{-1}\ cm^{-1}$ (*36*); imipenem, $\Delta\epsilon_{300} = 9000\ M^{-1}\ cm^{-1}$ (*37*). k_{cat}/K_M was estimated by nonlinear fitting of the data to an exponential decay, as the substrate concentrations used were less than 10% of previously reported K_M values for WT *B. cereus* βLII (*37*).

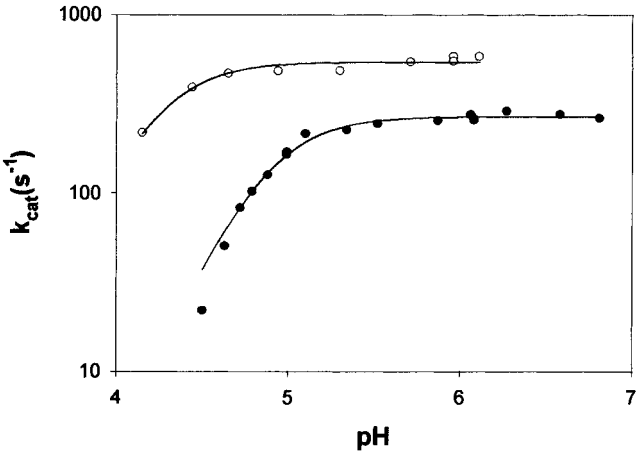

FIGURE 2: pH dependence of k_{cat} for benzylpenicillin hydrolysis by wild-type βLII. Data points were obtained in TACS buffer with 50 μg/mL BSA added, in the presence of 20 μM (closed circles) or 1 mM (open circles) zinc(II). The standard deviation of each data point is below 5%. Solid lines correspond to the data fit to eq 3, with pK_a values of 4.9 for 20 μM zinc(II) and 4.2 for 1 mM zinc(II).

RESULTS

pH Dependence of the Enzymatic Activity. The different binding affinities of the two zinc(II) equivalents may be exploited to study separately the kinetic behavior of the mono- and bi-zinc forms of the enzyme by regulating the zinc(II) concentration in the reaction milieu (*15*, *16*). Knowledge of the binding constants of the two metal binding sites allows to calculate the concentration of the three possible species (apoenzyme, mono-zinc, and bi-zinc) that coexist in equilibrium at a certain zinc(II) concentration. These constants can be estimated by measuring the β-lactamase activity at increasing zinc(II) concentrations added to the apoenzyme (*15*, *16*). For our purposes, these apparent dissociation constants are representative of the zinc(II) content on the enzyme during turnover, and they can be used to compare relative affinities for zinc(II). The chosen substrate for these experiments was benzylpenicillin, since the difference of apparent K_D values for this substrate is large enough to allow a separate kinetic characterization of the mononuclear and the binuclear species. Moreover, even if the activity enhancement by uptake of the second Zn(II) equivalent is modest, it can be better detected than toward other substrates (*16*).

At 20 μM zinc(II), the enzyme is mostly present (95%) in the mononuclear form, and the amount of binuclear derivative is negligible (2%). The 3% of inactive apoenzyme is not expected to interfere with the measurements. Instead, at 1 mM zinc(II), 54% of the enzyme is found in the dinuclear form. By adding further zinc(II), there is a nonnegligible hydrolysis catalyzed by the zinc(II)−hexaaquo complex. For this reason, the kinetic data retrieved at 1 mM zinc(II) were taken as the best approximation to evaluate the activity of the binuclear species. As a consequence, the catalytic efficiency of the binuclear species will be underestimated.

The time-course kinetics of benzylpenicillin hydrolysis were followed at different pH values ranging from 4.2 to 7.0 with different added concentrations of zinc(II) (Figure 2). The pH dependence of the penicillinase activity at 20

Table 1: Zinc(II) Content of Wild-Type βLII[a]

Zn(II) concentration (μM)	Zn/enzyme	
	pH 5.0	pH 6.0
0	0.23	1.01
0.2	0.25	0.99
20	0.88	1.10
100	1.06	1.13

[a] Enzyme concentration was ca. 50 μM. Metal content was determined after five dialysis steps against TACS buffer with the corresponding zinc(II) concentration over 24 h. Zn/enzyme was calculated from the differences of zinc(II) concentration between the enzyme sample and the dialysis buffer.

μM zinc(II) shows an acidic limb with a pK_a of 4.9, i.e., almost 1 unit of pH lower than the value reported by Bounaga et al. for the same enzyme (5.8) (*29*). The pH-induced inactivation is due to a falloff in k_{cat} (Figure 2), since K_M values are virtually pH-independent (see Figure S1, Supporting Information, for k_{cat}/K_M values). At 1 mM zinc(II), the activity decay is clearly shifted to lower pH values (Figure 2). An upper limit of 4.2 can be estimated for the pK_a under these conditions. At all the zinc(II) concentrations studied, the slope of the acidic decay of k_{cat} plotted in a logarithmic scale was 2, as pointed out by Bounaga et al. (*29*). This strongly suggests that the acidic inactivation exhibiting different pK_a values corresponds to the same event.

These results may suggest a prima facie that uptake of a second zinc(II) equivalent contributes to lowering the pK_a of the zinc-bound water. A pH profile performed at 100 μM added zinc(II) exhibited a pK_a of 4.7, i.e., intermediate to those found at 20 μM and 1 mM zinc(II). This is in line with the higher pK_a found by Bounaga et al. at even lower zinc(II) concentrations (0.4−20 μM) (*29*). One possible explanation to these observations is to assume that the enzyme inactivation at low pH is due to the reversible zinc(II) loss from the active site. Moreover, the enzyme activity of a sample incubated at low pH and 20 μM zinc(II) is fully recovered by adding extra zinc(II). Titration of the apoenzyme toward zinc(II) at pH 5.0 reveals uptake of only one zinc(II) equivalent, with a sensibly reduced affinity compared to data obtained at pH 6.0 (Figure S2, Supporting Information).

We also explored zinc(II) dissociation in the resting state enzyme by measuring the zinc(II) content of βLII at different pH values using atomic absorption spectroscopy. The data reported in Table 1 confirm that the binding affinity toward the first zinc(II) equivalent is impaired at acidic pH. The higher metal content with increasing zinc(II) concentrations correlates with the activity data at acidic pH (Figure S2, Supporting Information). This conclusion is consistent with data reported by Paul-Soto et al., in which a zinc(II):enzyme ratio of 0.6 was observed at pH 5.6 at 13.3 μM added zinc(II) (*16*).

The present data confirm that binding of both zinc(II) equivalents is not as tight as observed in most zinc enzymes, or in other metallo-β-lactamases, such as CcrA, IMP-1, and L1 (*17*, *21*−*23*). As already noted, the coordination environment of the two zinc(II) ions in βLII is identical to that found in CcrA and IMP-1 (*18*, *19*, *24*). One possible rationale for these strikingly different binding affinities is the presence of Arg121 near the active site (*18*, *24*, *25*).

Table 2: Catalytic Efficiency of Wild-Type and R121C βLII against Different β-Lactams[a]

substrate	wild type	R121C
benzylpenicillin	$(3.6 \pm 0.4) \times 10^5$	$(1.7 \pm 0.4) \times 10^5$
ampicillin	$(8.2 \pm 0.6) \times 10^4$	$(8.4 \pm 0.6) \times 10^4$
nitrocefin	$(3.2 \pm 0.1) \times 10^6$	$(2.8 \pm 0.7) \times 10^6$
cefotaxime	$(1.2 \pm 0.1) \times 10^6$	$(1.54 \pm 0.04) \times 10^6$
imipenem	$(1.64 \pm 0.04) \times 10^5$	$(2.36 \pm 0.06) \times 10^5$

[a] k_{cat}/K_M values ($M^{-1}\cdot s^{-1}$) determined in TACS buffer, pH 6.0 at 20 μM zinc(II) concentration. Reported values and standard deviations are the average of at least three independent determinations.

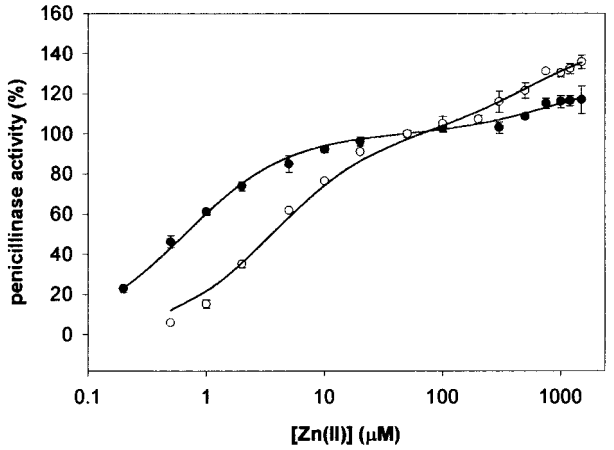

FIGURE 3: Titration of the apoenzymes vs zinc(II) followed by benzylpenicillinase activity for wild-type (closed circles) and R121C (open circles) βLII. The hydrolysis rate was determined in TACS at pH 6.0, and 50 μg/mL BSA. Rates are expressed in percent activity with 100% corresponding to the activity of the mononuclear enzymes. Solid lines were calculated using eq 1 with the following values: $K_{D1(WT)} = 0.66$ μM; $K_{D2(WT)} = 890$ μM; $K_{D1(R121C)} = 3.6$ μM; $K_{D2(R121C)} = 570$ μM.

Metal Binding Affinity. To explore the structural reasons for the weak binding of both zinc(II) ions to βLII, Arg121 was mutated by a Cys residue. This was performed by oligonucleotide-directed mutagenesis on single-stranded DNA (*32*). The mutant enzyme (R121C βLII hereafter) was expressed in *E. coli* as a fusion protein with glutathione-*S*-transferase (*15*, *33*) and purified in a soluble form with high yields. R121C βLII was able to hydrolyze benzylpenicillin, ampicillin, cefotaxime, imipenem, and nitrocefin (Table 2). Incubation of the mutant enzyme with EDTA rendered an inactive apoenzyme, as observed for wild-type βLII (*11*, *15*). The holoenzyme could be reconstituted by addition of zinc(II), recovering almost 100% of the catalytic activity observed prior to metal depletion. Addition of cobalt(II) to the apoenzyme gives rise to an active derivative, as observed for the native enzyme (*11*, *15*).

The metal binding capabilities of the mutant were examined by measuring the activity recovery of the corresponding apoenzyme in the presence of increasing concentrations of zinc(II). As shown in Figure 3, the two zinc(II) equivalents are sequentially bound with different affinities both in wild type and in R121-βLII. Moreover, the binding affinity toward the first zinc(II) ion is 6-fold reduced in the mutant enzyme (cf. Figure 3 caption). The uncertainty in the calculation of K_{D2} is clearly higher due to the small effect of the second zinc(II). However the data clearly show that binding of zinc(II) is similar in wild-type and R121-βLII. The binding affinity toward cobalt(II) was followed by fluorescence

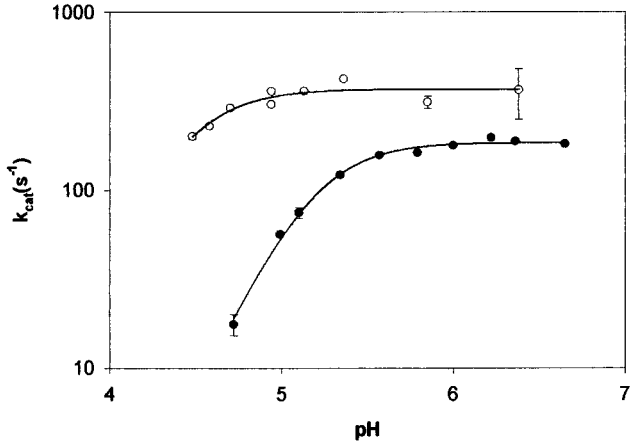

FIGURE 4: pH dependence of k_{cat} for benzylpenicillin hydrolysis by R121C βLII. Data points were obtained in TACS buffer with 50 μg/mL BSA added, in the presence of 20 μM (closed circles) or 1 mM (open circles) zinc(II). The standard deviation of each data point is below 5%, unless an error bar is indicated. Solid lines correspond to the data fit to eq 3, with pK_a values of 5.2 for 20 μM zinc(II) and 4.5 for 1 mM zinc(II).

spectroscopy, and was clearly impaired in the mutant enzyme as well (not shown). These results strongly suggest that the presence of Arg121 is not giving rise to an impaired zinc binding in βLII.

The R121C mutation is not expected to alter the metal site geometry. However, Arg121 is involved in the hydrogen bond network in the active site (cf. Figure 1A), and may affect the conformation of the metal ligands. To discard any structural change in the metal binding site, we obtained the Co(II),Co(II)-derivative of βLII R121C. The intensity and position of the ligand field transitions and of the charge transfer band of the cobalt enzyme are highly sensitive to changes in the geometry of the metal binding site (*15*, *38*). The electronic spectrum of Co(II),Co(II)-R121C is identical to that of the wild-type enzyme, thus indicating that the mutation has not altered the metals' coordination sphere.

Catalytic Performance of R121C βLII. The zinc binding experiment reveals also the effect of each zinc(II) equivalent on the penicillinase activity of the wild-type and R121C βLII (Figure 3). In both cases, uptake of the first zinc(II) gives rise to an active enzyme, but the incorporation of the second zinc(II) equivalent produces a stronger activating effect in the mutant (Figure 3).

The distinct penicillinase activities of the mutant enzyme with different zinc contents prompted us to study the kinetic behavior of the mono- and bi-zinc(II) adducts of R121C βLII, and their pH-dependence compared to wild-type βLII. These experiments were performed at 20 μM zinc(II) (where 82% of the R121C βLII is present as the mono-zinc derivative), and 1 mM zinc(II) (with 64% of the enzyme as the bi-zinc species). The activity decay at low pH occurs with pK_a values of 5.2 [20 μM zinc(II)] and 4.5 [upper limit at 1 mM zinc(II)], i.e., at higher pH values than in wild-type βLII (Figure 4). This information, together with the finding that R121C βLII binds Zn_1 with a lower affinity than the native enzyme, further supports the hypothesis that the inactivation at low pH is due to zinc dissociation from the active site. The activity falloff at low pH occurs in both cases with a slope of 2.0. As observed for the wild-type enzyme, K_M values are pH-independent.

Table 3: Kinetic Parameters for Benzylpenicillin Hydrolysis in TACS, pH 6

	$k_{cat(exp)}$ (s^{-1})[a]	$k_{cat(calc)}$ (s^{-1})[b]	K_M (μM)	k_{cat}/K_M ($M^{-1}\cdot s^{-1}$)
mono-zinc(II) WT	390 ± 10	390	1090 ± 80	(3.6 ± 0.4) × 10^5
mono-zinc(II) R121C	220 ± 30	250	1400 ± 200	(1.7 ± 0.4) × 10^5
bi-zinc(II) WT	600 ± 30	770	1900 ± 200	(3.2 ± 0.6) × 10^5
bi-zinc(II) R121C	490 ± 40	630	3300 ± 400	(1.5 ± 0.3) × 10^5

[a] Experimental k_{cat} values, as measured at 20 μM and 1 mM zinc(II) concentration, taken as representatives of the activity of the mono- and bi-zinc(II) species, respectively. Reported values and standard deviations are the average of at least three independent determinations. [b] Calculated k_{cat} values for the isolated mono- and bi-zinc(II) species.

The pH-independent kinetic parameters are summarized in Table 3. The k_{cat} value of mono-zinc(II) R121C βLII at pH 6.0 is reduced by 40% compared to wild-type βLII. However, the turnover number of R121C βLII at high zinc(II) concentrations is only 20% smaller than WT βLII.

DISCUSSION

pH-Dependence. Bounaga et al. have reported the pH dependence of k_{cat}/K_M for wild-type βLII at low zinc(II) concentrations (0.4−20 μM), concluding that protonation of two groups contributes to enzyme inactivation at acidic pH (*29*). These residues were tentatively identified as the zinc-bound hydroxide and Asp120. The present data demonstrate that this inactivation is due to an decrease in k_{cat}, that we attribute to zinc(II) dissociation at acidic pH. The addition of further zinc(II) at low pH immediately gives rise to a fully active enzyme. Atomic absorption measurements indicate that zinc(II) loss occurs as well in the free enzyme at low pH. Hence, the lower pK_a values observed at higher zinc(II) concentrations reflect shifting of this equilibrium. R121C displays a lower zinc binding affinity than wild-type βLII, thus experiencing zinc(II) dissociation at higher pH values. The slope of the acidic limb is 2.0 in all cases, thus suggesting that two protonating groups with close pK_a values are involved in the zinc(II) dissociation process. These residues may probably be two of the His ligands binding Zn_1, which is mostly responsible for the activity of the enzyme.

These conclusions indicate that, provided there is enough zinc(II) available to occupy the first binding site, there are no protonating processes in the active site with pK_a values >4.9 able to affect the enzyme efficiency. This picture is similar to that observed in the binuclear enzyme CcrA, in which the zinc-bound water is presumed to exhibit a pK_a lower than 5.2 (*31*). In addition, the higher zinc(II) binding affinity in CcrA may prevent zinc(II) dissociation at low pH values. This is supported by very recent results that show that the activity of the monozinc(II) derivative of the C121R mutant of CcrA lacks the pH dependence observed in βLII (*17*). Therefore, the pK_a values of the zinc-bound water in mono-zinc(II) wt and R121C βLII are low enough to allow deprotonation at physiological pH. The crystal structures of different metallo-β-lactamases reveal that the Zn_1−O bond length is as short as 1.9 Å (*18*, *19*, *24*), giving support to the hypothesis that a hydroxide (and not a water molecule) is bound at neutral pH. Arg121, being involved in the

hydrogen bond network of this water molecule, may influence its pK_a. The present data indicate that the water pK_a in R121C βLII is <5.2. Hence, replacement of Arg121 does not significantly increase this pK_a.

Two major conclusions may be derived from this analysis: (1) the pK_a of the zinc-bound water in mono-zinc(II) lactamases is below 4.9, and consequently (2) binding of a second zinc(II) ion is not needed for nucleophile activation in metallo-β-lactamases. Hence, once the active form of the nucleophile (a metal-bound hydroxide) is formed, coordination to another zinc(II) ion would result in a decrease in nucleophilicity, as concluded from recent model complex studies from Kaminskaia et al. (*39*, *40*). These conclusions do not conflict with the presence of a second zinc(II) ion in other lactamases. The mechanism recently proposed by Wang et al. for the binuclear enzyme considers detaching of the bound hydroxide to Zn_2 prior to the nucleophilic attack (*30*), as proposed also for *A. proteolytica* aminopeptidase (*41*) and phosphotriesterase (*42*). The bridged and unbridged forms of the bi-zinc enzyme could be in equilibrium in the resting state form. Carfi et al. have reported a structure of CcrA that lacks any solvent molecule bridging the two zinc(II) ions (*43*). Recent theoretical calculations on the *B. fragilis* enzyme reveal a flat potential surface that allows the coexistence of different conformations of the binuclear zinc(II) site (*44*).

A pK_a value below 4.9 for a $His_3Zn(Wat)$ coordination environment contrasts with the pK_a = 6.8 reported for carbonic anhydrase, with an identical coordination polyhedron (*45*). However, this situation is not unprecedented. A similar situation has been met in matrilysin, with a similar coordination site, and a pK_a of 4.3 (*46*). Metal-bound pK_a values in zinc hydrolases are determined by direct and second-shell ligands (*47*−*49*). Zinc(II) binding favors deprotonation of the imidazole group of zinc(II) ligands, even toward carboxylate groups. In carbonic anhydrase, the His ligands are involved in strong hydrogen bond interactions with a glutamic acid ($r_{N\cdots O}$ = 2.5 Å) and two side-chain amide groups ($r_{N\cdots O}$ = 2.7 Å) (*50*). Instead, in βLII, the hydrogen bonds are clearly weaker: a more distant Asp side chain ($r_{N\cdots O}$ = 3.0 Å) and two water molecules ($r_{N\cdots O}$ = 2.7 and 2.8 Å) fulfill this role (*24*) (Figure S3, Supporting Information). Theoretical calculations on liver alcohol dehydrogenase show that a strong imidazolate carboxylic acid hydrogen bond polarizes the histidine ligand, strenghtening the histidine−zinc(II) bond and decreasing the positive charge on the zinc(II) ion.[2] Hence, in βLII the weaker interaction with the second-shell ligands should increase the positive charge on the zinc(II) ion, enhancing the acidity of the bound water (*51*, *52*).

A highly acidic metal-coordinated water reflects a strong Lewis acidity of the zinc(II) ion, thus being able to generate a high concentration of available hydroxide in the active site (*41*, *53*−*55*). However, the stronger the Lewis acidity of zinc(II), the weaker the nucleophilicity of the coordinated hydroxide. This may indicate that there is no functional rationale for such a low bound-water pK_a. The mechanism proposed by Bounaga et al. for the mono-zinc(II) enzyme has raised the possibility of a dianionic intermediate (Figure 5)(*29*). An increase of the positive charge in the zinc(II) ion

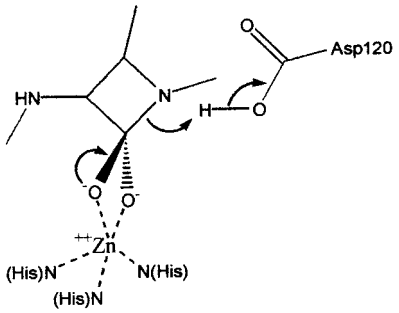

FIGURE 5: Schematic drawing of the proposed dianionic intermediate in the reaction mechanism of βLII (*29*).

may certainly help in stabilizing this intermediate. The kinetic data from the R121C mutant indicate that the presence of a positive charge in the active site enhances the catalytic efficiency (see below), giving support to this suggestion, that still needs direct evidence.

Role of Arg121 in Zinc Binding and Catalysis. Replacement of the positively charged Arg121 located near the active site does not give rise to a tightly bound binuclear site in βLII (Figure 3). This allows us to discard the hypothesis that this residue impairs metal binding in the wild-type enzyme through electrostatic repulsion, as suggested (*18*, *24*, *25*). Recently, Fast et al. obtained the C121R mutant in the binuclear CcrA enzyme, that recreates the Arg121 residue present in βLII (*17*). Although removal of the second zinc(II) equivalent proved to be easier in this mutant than in the wild-type enzyme, the mutant was isolated in the bi-zinc form (*17*, *23*). This indicates that the arginine residue may affect binding in the nanomolar range, but not to an extent to abolish in vivo binding of Zn_2, as happens in βLII.

Two metallo-β-lactamases seem to be functional in vivo as mononuclear enzymes: CphA from *A. hydrophila* (*14*) and βLII from *B. cereus* (*15*, *56*), and both possess an arginine residue in position 121 (*57*, *58*). Arg121 is also conserved in the enzymes VIM-1 from *P. aeruginosa* (*59*), BlaB from *C. meningosepticum* (*60*), and IND-1 from *C. indologenes* (*61*). When not present, Arg121 is replaced by small polar residues (Cys and Ser in the CcrA and IMP-1 enzymes, respectively) located beyond the metals' coordination sphere (*18*, *19*), or by a His residue, that becomes a Zn_2 ligand in the enzyme L1 from *S. maltophilia* (*20*). This suggests that the presence of an arginine residue in position 121 could be a common feature of mononuclear metallo-β-lactamases (Figure S4, Supporting Information). It is interesting to note that the mono-zinc(II) derivative of *B. fragilis* CcrA obtained in vitro is almost as active as the bi-zinc(II) form (*16*). However, the high affinity of this enzyme toward both zinc(II) ions suggest that the active form in vivo may be the binuclear one.

The kinetic parameters estimated at 20 μM zinc(II) are expected to reflect mostly the activity of the mono-zinc(II) form, that represents ca. 90% of the total enzyme. However, the values obtained at 1 mM zinc(II) underestimate the catalytic efficiency of the bi-zinc(II) enzyme. By assuming that the k_{cat} values are a weighted average of the contributions of the mono- and bi-zinc(II) species, we calculated the theoretical k_{cat} of the pure mono- and bi-zinc(II) forms for both enzymes (Table 1). By comparing these values, we observe that mutation of Arg121 reduces the k_{cat} toward benzylpenicillin of the mono-zinc(II) species from 390 to

[2] Gervasio et al., unpublished results.

250 s^{-1}. Uptake of a second zinc(II) equivalent induces a larger k_{cat} increase in R121C, as qualitatively observed in the titration experiment (Figure 3), that almost parallels that measured for the bi-zinc(II) wild-type enzyme (Table 3). This indicates that the impaired activity in the R121C mutant can be mostly restored by uptake of a second zinc(II) equivalent. The ratio of k_{cat} values for the mono- and bi-zinc(II) species is 2.0 in WT βLII, and 2.5 in R121C βLII (Table 3). In other words, the presence of Zn_2 is more significant for benzylpenicillinase activity in R121C than in the wild-type enzyme.

A rationale for the presence of Arg121 in mononuclear-β-lactamases may be attempted as follows. The metal-bound hydroxide/water in zinc hydrolases is optimally oriented for nucleophilic attack to the substrate through hydrogen bond networks (*62*, *63*). In βLII, this role is fulfilled by Asp120, that is hydrogen bonded to Arg121 (Figure 1A). The lower activity of mono-zinc(II) R121C may be explained by assuming that Asp120 is in a looser conformation. Instead, in typical binuclear enzymes (such as CcrA), Asp90 could be anchored by coordination to Zn_2, thus mimicking the role of Arg121 in the mononuclear enzyme (Figure 1B). The increase in activity seen when going from mononuclear to binuclear R121C may be accounted for in this way. DFT calculations suggest that the absence of Arg121 in the mono-zinc(II) species may affect the position and protonation state of Asp90.[3] These observations are in line with results in the C221S mutant of βLII, in which the hydrogen bond interaction between Arg121 and Asp120 is altered, affecting the catalytic efficiency of the enzyme (*64*). These results altogether highlight the relevance of this hydrogen bond network in mono-zinc(II) β-lactamases.

After submission of this paper, a study on βLII was published (*65*). In this work, the authors suggest that the mononuclear species in βLII contains one zinc equivalent distributed equally between both binding sites. Our data can be framed into this picture by assuming that the first metal binding site is responsible for the catalytic activity. Hence, removal of arginine 121 might favor the occupancy of the second site in the mono zinc(II) derivative, impairing its catalytic performance. Uptake of two zinc(II) equivalents would thus allow the enzyme to regain activity.

CONCLUDING REMARKS

We have clearly demonstrated that the reversible inactivation at a low pH of βLII is due to zinc(II) dissociation from the active site. As a consequence, it is inferred that the zinc-bound water in mono-zinc(II) βLII possesses a $pK_a < 4.9$, and this result may be extrapolated to other lactamases. This reveals that the second zinc(II) ion in binuclear lactamases is not necessary for lowering the pK_a of the nucleophile. A possible role for Zn_2 as being part of a network aimed to orient the essential residue Asp120, and, ultimately, the attacking nucleophile, is proposed from the behavior of the R121C mutant. β-Lactamases from different organisms may utilize alternatively Arg121 or a second zinc(II) equivalent in a modular way with this purpose. In binuclear enzymes, Zn_2 could then be considered as a cocatalytic metal site, according to the classification proposed by Auld and Vallee (*66*).

[3] Dal Peraro et al., unpublished results.

The low binding affinity of both zinc(II) ions in βLII is not due to the presence of Arg121, as revealed by the study of R121C βLII. It is noteworthy pointing out that the dissociation constants for Zn_1 of the free enzyme [0.33 μM for the *B. cereus* 5/B/6 β-lactamase II (*16*)] are high compared to those normally observed in zinc enzymes [e.g., 7 pM for *A. hydrophila* metallo-β-lactamase (*67*) and 4 pM for human carbonic anhydrase II (*68*)]. Under these terms, βLII would not fulfill the minimal requirements to be considered a metalloenzyme. Notwithstanding, the basic structural features required for β-lactamase activity (such as preformed hydroxide) are already well-defined in βLII. Other enzymes, such as CcrA, IMP-1, or L1 (or even the mononuclear CphA), may possess more exquisite features shaped by different evolutionary pressures that still need to be explored.

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SUPPORTING INFORMATION AVAILABLE

pH dependence of k_{cat}/K_M for benzylpenicillin hydrolysis by wild-type and R121C βLII; benzylpenicillinase activity of βLII vs zinc(II) concentration at pH 5 and 6; schematic representation of the active sites of human carbonic anhydrase(II) and βLII, and sequence alignment of metallo-β-lactamases. This material is available free of charge via the Internet at http://pubs.acs.org.

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