Zn(II) Dependence of the *Aeromonas hydrophila* AE036 Metallo-β-lactamase Activity and Stability[†]

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ABSTRACT: Two Zn^{2+} binding sites were found in the *Aeromonas hydrophila* AE036 metallo-β-lactamase. The affinity of the first binding site for Zn^{2+} ions is so high that the dissociation constant could not be determined, but it is significantly lower than 20 nM. The mono-Zn^{2+} form of the enzyme exhibits a maximum activity against its carbapenem substrates. The presence of a Zn^{2+} ion in the second lower affinity binding site results in a loss of enzymatic activity with a K_i value of 46 μM at pH 6.5. The kinetic analysis is in agreement with a noncompetitive inhibition mechanism. The Zn content of the *A. hydrophila* enzyme is also strongly pH-dependent. With an external Zn^{2+} ion concentration of 0.4 μM, occupancy of the higher affinity site by metal ions is lower than 10% at pH 5 and 10. The affinity for the second binding site seems to increase from pH 6 to 7.5. Fluorescence emission and circular dichroism spectra revealed slight conformational changes upon titration of the apoenzyme by Zn^{2+} ions, resulting in the successive saturation of the first and second binding sites. Differential scanning calorimetry transitions and intrinsic fluorescence emission spectra in the presence of increasing concentrations of urea demonstrate that the catalytic zinc strongly stabilizes the conformation of the enzyme whereas the di-Zn enzyme is even more resistant to thermal and urea denaturation than the mono-Zn enzyme. The Zn^{2+} dependency of the activity of this metallo-β-lactamase thus appears to be very different from that of the homologous *Bacteroides fragilis* enzyme for which the presence of two Zn^{2+} ions per molecule of protein appears to result in maximum activity.

Class B β-lactamases (Ambler, 1980; Bicknell et al., 1985; Frère, 1995) require divalent metal ions (most often Zn^{2+}) as enzymatic cofactors. By catalyzing the hydrolysis of the β-lactam amide bond, they protect bacteria against the lethal effects of this group of antibiotics.

The 3D structures of the *Bacillus cereus* 569H (Carfi et al., 1995) and *Bacteroides fragilis* CcrA (Concha et al., 1996) metallo-β-lactamases have recently been solved at pH 5.6 and 7.0, respectively, revealing the presence of one and two Zn ions in the active sites of the former and the latter, respectively. Both Zn ions appeared to be tightly bound in the *Bacteroides* enzyme, and the catalytic activity was correlated to the occupancy of both sites (Crowder et al., 1996), while in the *B. cereus* enzyme a second binding site of significantly lower affinity was also present whose occupancy merely increased the enzymatic efficiency (Baldwin et al., 1978; Carfi et al., 1997). Similarly, the addition of Zn ions to the buffers has been found to activate or stabilize various metallo-β-lactamases (Payne et al., 1994). As a consequence, the exact stoichiometry of the metal requirement has become a major question in the analysis of the catalytic mechanism of metallo-β-lactamases.

Three metallo-β-lactamases produced by various *Aeromonas* strains, potentially pathogenic for man, have been described. The enzymes from *Aeromonas hydrophila* AE036 (Felici et al., 1993; Felici & Amicosante, 1995; Segatore et al., 1993), from *Aeromonas sobria* 163a (Walsh et al., 1996), and from *Aeromonas jandaei* (previously called *Aeromonas sobria*) AER 14M (Youjun & Bush, 1996) are characterized by similar and very peculiar specificity profiles. They effectively hydrolyze carbapenems and show poor activity against penicillins and cephalosporins, a behavior in contrast to that of other metallo-β-lactamases which usually exhibit a very wide activity spectrum against most β-lactam compounds with the exception of monobactams. Moreover, the *A. jandaei* AER 14M enzyme has been studied in essentially Zn-free buffer since the metal acts a strong inhibitor (Youjun & Bush, 1996).

In this study, we have analyzed the metal dependency of the *A. hydrophila* AE036 metallo-β-lactamase activity and determined the Zn^{2+}/enzyme stoichiometry under various pH and ionic strength conditions. The enzyme binds a first Zn ion very tightly (K_d < 20 nM), which results in optimal activity. The binding of the second Zn ion noncompetitively

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inhibits the enzyme with a K_i value of 46 μM at pH 6.5. The metal content also significantly influences the stability of the protein.

MATERIALS AND METHODS

Metal-Free Buffers. The metal-free buffers were obtained by vigorous stirring of bidistilled water with 0.2% (v/v) cross-linked agarose−iminodiacetic acid (IDA; Affiland, Liège, Belgium). Plasticware was successively washed with 20% HNO_3 and metal-free water before use. Metal-free dialysis tubing was prepared as described by Auld et al. (1988). Even after treatment, the "metal-free" buffers still contained about 20 nM Zn^{2+} as determined by ICPMS (see below). Non-treated buffers, prepared in bidistilled water, contained higher concentrations of contaminating Zn^{2+} ions, about 0.4 μM for the cacodylate and Hepes buffers.

Enzyme Purification. The *Aeromonas hydrophila* AE036 metallo-β-lactamase was overexpressed and purified as previously described (Hernandez et al., 1996).

In the present study, the periplasmic fractions and enzyme solutions were prepared in 15 mM sodium cacodylate buffer, pH 6.5, prepared with milliQ water. An additional purification step was carried out by affinity chromatography. A column of IDA−agarose was modified to improve its resolution (Felici et al., 1993). The protein was loaded onto the column after equilibration with 15 mM sodium cacodylate buffer, pH 6.5. The elution was carried out by a linear NaCl gradient (0−0.2 M) in 10 column bed volumes of the same buffer. The fractions containing β-lactamase activity were pooled, concentrated, and dialyzed against the same salt-free buffer. The enzyme was stored at −20 °C, and no activity was lost after prolonged storage (up to 12 months) under these conditions. The purified protein was analyzed by mass spectrometry in a VG Platform FISONS spectrometer under denaturing conditions (50% v/v acetonitrile and 0.1% v/v formic acid). All spectra were scanned with a flow rate of 40 μL/min, and with a cone voltage of 40 V.

Inactivation of the Metallo-β-lactamase by Chelating Agents. The progressive inactivation of the enzyme (0.9 nM) by ethylenediaminetetraacetic acid (EDTA), ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1,10-phenanthroline, and pyridine-2,6-dicarboxylic acid (dipicolinic acid) was monitored in the presence of 200 μM imipenem in a total volume of 0.5 mL of 15 mM sodium cacodylate buffer, pH 6.5, at 25 °C (reporter substrate method; De Meester et al., 1987).

The rate constants characterizing the inactivation of the enzyme were derived from the dependence of the pseudo-first-order inactivation rate constant, k_i, upon the chelating agent concentration, on the basis of Scheme 1 where E·Zn^{2+}, C, E·Zn^{2+}·C, E, and Zn^{2+}·C are the metalloenzyme, the chelator, a ternary metalloenzyme−chelator complex, the apoenzyme, and the metal−chelator complex, respectively. *K* represents the dissociation constant of the ternary complex E·Zn^{2+}·C, k_{+2} is the individual rate constant for the dissociation, and k_{-2} is the second-order individual constant for the formation of the ternary complex, respectively.

Scheme 1

$$\text{E.Zn} + \text{C} \underset{}{\overset{K}{\rightleftharpoons}} \text{E.Zn.C} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \text{E} + \text{Zn.C}$$

As [Zn^{2+}·C] was higher than [E], the formation of the ternary complex from apoenzyme and the Zn−chelator complex was characterized by the pseudo-first-order rate constant $k_{-2}' = k_{-2}$[Zn·C] (Scheme 1).

The values of the individual rate constants were obtained with the help of eq 1 where K_m^S and [S] are respectively the K_m and the concentration of the reporter substrate.

$$k_i = \frac{k_{+2}[C]}{K[(K_m^s + [S])/K_m^s] + [C]} + k_{-2}' \qquad (1)$$

When the *K* value is much larger than the chelator concentration, eq 1 simplifies to

$$k_i = \frac{k_{+2}[C]K_m^s}{K(K_m^s + [S])} + k_{-2}' \qquad (2)$$

Apoenzyme Preparation. The Zn^{2+}-free enzyme was obtained by dialysis of a 500 μM solution against 100 volumes of 10 mM EDTA in 15 mM sodium cacodylate metal-free buffer, pH 6.5, at 4 °C. The enzyme activity was tested by monitoring the hydrolysis of 200 μM imipenem. When the residual activity was lower than 1%, the metal-free enzyme was dialyzed against the same volume of 15 mM sodium cacodylate metal-free buffer, pH 6.5, at 4 °C. The buffer was changed 5 times, and the duration of each dialysis was about 8 h. In a parallel experiment, the enzyme was incubated in metal-free buffer containing 10 mM ^{14}C-EDTA (5 mCi $mmol^{-1}$, Sigma) for 2 days at 4 °C. The radioactive chelating agent was eliminated as above and in the presence of 1 M NaCl in the same buffer. The radioactivity of an aliquot was determined after each change of dialysis buffer. The residual radioactiviy was measured by liquid scintillation.

Zn^{2+}-Dependence of the Aeromonas hydrophila Metallo-β-lactamase Activity. The enzyme activity was determined by monitoring the initial rate of hydrolysis of 210, 150, and 90 μM imipenem at 25 °C in 15 mM sodium cacodylate buffer, pH 6.5, containing increasing concentrations of $ZnSO_4$. The diluted enzyme solutions were prepared with buffers containing 50 μg mL^{-1} bovine serum albumin.

Determination of the Metal Ion Content of the Metallo-β-lactamase. Particle-induced X-ray emission (PIXE) (Robaye et al., 1981; Johansson & Campbell, 1988) and inductively coupled plasma mass spectrometry (ICPMS) were used to determine the metal content of the apo-β-lactamase in buffers and in the metallo-β-lactamase solutions. In the latter case, all determinations were done after an overnight dialysis at 4 °C against 250 volumes of the same buffer containing increasing concentrations of $ZnSO_4$. The enzyme concentration was 160 μM and 30 μM in the PIXE and ICPMS assays, respectively. In PIXE experiments, the target solution was mixed with an equal volume of a 1000 ppm yttrium solution as an internal monitor for normalizing measurements. A LeGe (low-energy Ge) detector was used instead of a Si(Li) detector in order to remove the As−Si escape peak interference due to the buffer.

The effect of pH on the binding of Zn to the metallo-β-lactamase was also studied by ICPMS. The following buffers were used (the concentration of the buffering component was 50 mM in all cases): citric acid/sodium citrate (pH 5); sodium cacodylate/HCl (pH 6, 6.5, and 7); HEPES/HCl (pH 7.5 and 8); and glycine/NaOH (pH 9 and 10), in the absence and presence of 100 μM $ZnSO_4$. The

Zn content was also determined in 15 mM sodium cacodylate buffer, pH 6.5, containing 0, 0.05, 0.1, 0.5, and 1 M NaCl, both in the absence and in the presence of 100 μM Zn^{2+}. The enzyme preparation was also analyzed for the presence of some other divalent metals (Cd^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Hg^{2+}, Ni^{2+}, and Ca^{2+}).

Structural Studies of Apo- and Metallo-β-lactamase at Different Zn^{2+} Concentrations. The *Aeromonas hydrophila* β-lactamase was first incubated at different temperatures in the absence and presence of 100 μM Zn^{2+}. Samples were withdrawn after increasing periods of time, and their enzymatic activity was assayed at 30 °C by determining the initial rate of 200 μM imipenem hydrolysis. Thermal inactivation of the apoenzyme after addition of 0, 0.67, and 1 equiv of Zn^{2+} and of 0.1 and 1 mM Zn^{2+} in 15 mM sodium cacodylate metal-free buffer and in untreated buffer, respectively, was carried out in a Microcal Inc. MCS differential scanning calorimetry (DSC) unit at a scan rate of 60 °C per hour. The MCS Observer software package was used for data acquisition and analysis. In all cases, the buffer−buffer base line was subtracted. All protein solutions and buffers were degassed for 3 min under vacuum with gentle stirring before being loaded into the calorimeter. The cells were carefully washed with metal-free water before analysis of the apoenzyme samples. The protein concentration was in the 70−110 μM range.

Fluorescence emission spectra of the enzyme in the presence of increasing amounts of Zn^{2+} were recorded at 25 °C on a Perkin Elmer LS50 luminescence spectrometer using excitation and emission wavelengths of 280 and 333 nm, respectively. The concentration of the apoenzyme (2 μM) was twice that of the holoenzyme in order to reduce the risk of metal contamination. The cuvette was washed with 20% HNO_3 prior to use. Urea denaturation studies of the β-lactamase in the absence and presence of 1 mM Zn^{2+} were also carried out. The apo, mono-Zn, and di-Zn enzymes were incubated for 16 h in increasing concentrations of urea (0−8 M) at 4 °C. Fluorescence emission spectra were obtained for all samples as described above.

Circular dichroism spectra of the enzyme were obtained using a Jobin Yvon CD6 spectrometer. The spectra were scanned at 25 °C with 1-nm steps from 202 to 260 nm.

Determination of the Kinetic Parameters. The hydrolysis of the antibiotics was monitored by following the absorbance variation resulting from the opening of the β-lactam ring. K_m and k_{cat} values were derived from complete hydrolysis time courses (De Meester et al., 1987) or initial rate measurements. The noncompetitive inhibition by excess Zn^{2+} ions was studied under initial rate conditions with imipenem concentrations in the 90−210 μM range and in 15 mM sodium cacodylate buffer, pH 6.5 at 25 °C. Substrate-induced inactivation was studied as described by De Meester et al. (1987). The stability of all antibiotics was also tested in the presence of the metal ion concentrations used for enzymatic assays. All experiments were performed at 25 °C.

Benzylpenicilloic acid was obtained by hydrolysis of benzylpenicillin at pH 12.

RESULTS AND DISCUSSION

Purification. The enzyme was 95% pure after the S-Sepharose FF step as judged by SDS−PAGE gel and mass spectrometry (Hernandez et al., 1996). However, after storage at 4 °C for 1 month, these preparations exhibited a decreased activity, and the presence of degraded protein was detected by SDS−PAGE analysis. This was probably due to the presence of traces of contaminating protease(s). After the affinity chromatography step on IDA−agarose, the solution was stable, and its purity was confirmed by mass spectrometry. The measured molecular mass (25 170 ± 15 Da) was in good agreement with that deduced from the nucleotide sequence (25 188 Da; Massidda et al., 1991).

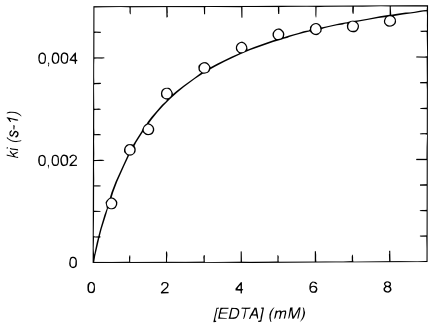


FIGURE 1: Pseudo-first-order inactivation rate constant (k_i) as a function of EDTA concentration. The line was drawn by fitting to the equation $k_i = a[\text{EDTA}]/(b + \text{EDTA})$ where $a = 5.8 \times 10^{-3}$ s^{-1} and $b = 1.5 \times 10^{-3}$ M. The reporter substrate was 200 μM imipenem, and the final enzyme concentration was 0.9 nM.

Inactivation by Zn-Chelating Agents. With the four tested chelating agents, a time-dependent pseudo-first-order inactivation was observed. The value of the pseudo-first-order rate constant k_i increased with the concentration of the chelating agent in a hyperbolic manner with EDTA (Figure 1), 1,10-*o*-phenanthroline, and dipicolinic acid and linearly with EGTA. These data indicate that the chelators do not act by scavenging the free metal and can be explained by the formation of a ternary enzyme−metal−chelator complex prior to the formation of the apoenzyme. With the three compounds for which a hyperbolic variation of k_i was observed, the curves were analyzed on the basis of eq 1 assuming that k_{-2}' was negligible. With EGTA, on the basis of eq 2, k_{-2}' was obtained by extrapolation of the line to zero EGTA concentration (Table 1).

The apoenzyme was prepared by dialysis against EDTA solutions. The ^{14}C-EDTA assay showed that the concentration of residual ^{14}C-EDTA after the fifth dialysis was 0.35% that of the enzyme and that no unwanted interaction took place between the chelating compound and the enzyme (Table 2).

The addition of 1 M NaCl improved the removal of ^{14}C-EDTA in the first and second dialysis steps. The apoenzyme was stored at −20 °C. Full enzymatic activity was recovered when 1 equiv of Zn^{2+} was added to the metal-free enzyme.

Zn^{2+}Dependence of the β-Lactamase Activity. When increasing Zn^{2+} concentrations were added to the monozinc enzyme, the initial hydrolysis rate of imipenem in the presence of increasing Zn(II) concentrations markedly decreased (Figure 2). The values of K_m and k_{cat} were determined in the absence and presence of 20, 50, and 100 μM Zn^{2+} to determine the type of inhibition. The results (Table 3) indicated a "pure" noncompetitive inhibition mechanism where K_m is equal to K_m', K_i is equal to K_i', and the activity is essentially zero at Zn^{2+} concentrations higher than 2 mM, indicating that the E·Zn_2S complex has little if any activity (Scheme 2, $k'_{cat} \ll k_{cat}$). Indeed, in the presence

Table 1: Individual Parameters for the Inactivation of the *A. hydrophila* AE036 Metalloenzyme by Chelating Agents[a]

chelating agent	K (μM)	k_{+2} (s^{-1})	k_{-2}' (s^{-1})	k_{+2}/K (M^{-1} s^{-1})
EDTA	1750 ± 100	6.00×10^{-3}	$<0.1 \times 10^{-3}$	3.40
EGTA	>1000	$>9 \times 10^{-2}$	4×10^{-3}	90
1,10-phenanthroline	100 ± 5	4.42×10^{-2}	$<0.2 \times 10^{-2}$	4.40×10^{2}
dipicolinic acid	5 ± 0.2	1.93×10^{-2}	$<0.2 \times 10^{-2}$	3.86×10^{3}

[a] The final enzyme concentration was 0.9 nM. Unless otherwise stated, SD values were <10%.

Table 2: ^{14}C-EDTA Content of Apoenzyme Preparation (500 μM) after Removal of the Chelator by Dialysis against 15 mM Sodium Cacodylate Buffer, pH 6.5[a]

no. of dialysis buffer	[^{14}C-EDTA]/[apoenzyme]		[EDTA] (μM)
	0 M NaCl	1 M NaCl	
0	20	20	10^4
1	1.5	0.54	
2	0.11	2.6×10^{-2}	
3	1.2×10^{-3}	9.5×10^{-4}	
4	4×10^{-4}	5.5×10^{-4}	
5	2.8×10^{-4}	4×10^{-4}	0.14/0.20

[a] For all data, the SD values were below 10% of the mean.

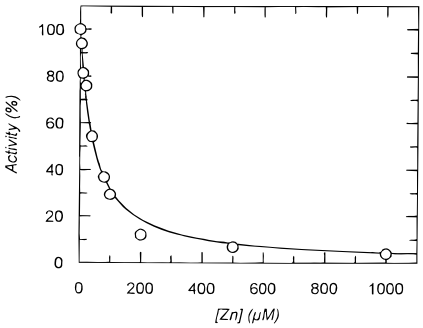


FIGURE 2: Plot of residual activity (%) of the *A. hydrophila* enzyme *versus* increasing Zn(II) concentrations. The line was obtained by fitting to the equation $100(v_i/v_0 = K_i/([Zn^{2+}] + K_i)$ where $K_i = 46 \pm 3$ μM. The points represent the average of measurements made with 90, 150, and 210 μM imipenem whose concentration did not significantly modify the v_i/v_0 ratios. The enzyme concentration was 0.6 nM in all cases.

Table 3: Kinetic Parameters for Hydrolysis of Imipenem by the *A. hydrophila* β-Lactamase (3 nM) at Different Zn Ion Concentrations[a]

[Zn^{2+}] (μM)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})
0	180	1350	7.5
20	170	850	5
50	180	480	2.7
100	170	360	2.1

[a] The residual Zn^{2+} concentration in the treated buffer was 20 nM, but the same k_{cat} and K_m values were obtained with untreated buffer whose contaminating Zn^{2+} concentration was 0.4 μM. For all data, the SD values were below 10% ($n = 4$).

of the highest concentrations of metal (>2 mM), spontaneous hydrolysis of imipenem was observed, and the addition of the enzyme did not significantly increase the hydrolysis rate (four independent experiments). The K_i value derived from the data presented in Figure 2 was 46 ± 3 μM. When protein samples containing up to 1 mM Zn^{2+} were dialyzed overnight against Zn-free buffer, the enzymatic activity was fully recovered, indicating the reversibility of the phenomenon.

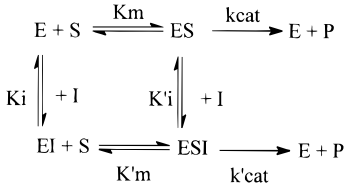
Metal Content. All class B β-lactamases require Zn ions for their activity. Recent crystallographic (Concha et al., 1996) and atomic absorption (Crowder et al., 1996) studies on the *Bacteroides fragilis* metallo-β-lactamase have shown

Table 4: Metal/Enzyme Ratio for the *A. hydrophila* β-Lactamase (pH 6.5)[a]

sample	[Zn^{2+}] in dialysis buffer (μM)	[Zn^{2+}]/[E] ratio		
		by ICPMS	by PIXE	estimated with $K_i = 46$ μM
enzyme	0 (0.4)[b]	0.9	1.0 ± 0.09	
enzyme	50	1.5 ± 0.1		1.52
enzyme	100	1.7 ± 0.1	1.8 ± 0.1	1.68
enzyme	200	1.9 ± 0.1		
enzyme	500		2 ± 0.1	1.90
enzyme	1000		2 ± 0.15	1.94
apoenzyme	0 (0.02)[b]		0.02	not applicable

[a] The enzyme concentration was 30 μM for ICPMS and 160 μM for PIXE measurements. The metal/enzyme ratio was calculated from the differences of Zn(II) concentration between the enzyme sample and the dialysis buffer at pH 6.5. Both techniques can be applied for metal determination when the ratio of metal concentration in the dialysis buffer to that of the enzyme concentration is not higher than 6. [n] As measured by ICPMS.

Scheme 2



that this enzyme binds 2 mol of Zn(II) per mole and that the presence of both metal ions appears to be required for full catalytic activity. By contrast, one single Zn(II) has been identified by crystallography (Carfi et al., 1995) in the *Bacillus cereus* metallo-β-lactamase at pH 5.6. As a consequence, the Zn content of the *A. hydrophila* enzyme was determined in the absence and presence of increasing concentrations of Zn(II) ions at pH 6.5 by PIXE and ICPMS (Table 4). Both techniques detected only one Zn ion per molecule of enzyme when the buffer did not contain added metal. Cd(II), Ca(II), Co(II), Cu(II), Mn(II), Hg(II), and Ni-(II) were not found in the protein samples.

After extensive dialysis against metal-free buffer, the enzyme still contained 1 equiv of Zn per mole and the apoenzyme immediately recovered maximal activity when diluted in metal-free buffer for the initial rate measurements. ICPMS showed that the "metal-free buffer" still contained about 20 nM Zn^{2+}, and it could be concluded that the dissociation constant was well below this value. Similarly, at a concentration of 30 μM, the apoenzyme contained a very low, but significant, concentration of Zn^{2+}(2%, Table 4), probably reflecting this contamination of the "metal-free buffers". The enzyme requires the presence of only one Zn ion for full activity and the activity of the di-Zn(II) enzyme against carbapenem antibiotics is negligible. Indeed, the loss of activity parallels the occupancy of the second binding site. As shown in Table 4, the K_D value of 46 ± 3 μM for the second Zn(II) was in good agreement with the occupancy

Table 5: Metal/Enzyme Ratio and Kinetic Parameters of the Metallo-β-lactamase at Different Zn Ion Concentrations and pH Values[a]

pH	[Zn^{2+}] in dialysis buffer (μM)	[Zn^{2+}]/[enzyme][b]	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
5	0.4	0.08 ± 0.01			600
5	100	0.1 ± 0.03			8×10^4
6	0.4	0.60 ± 0.05	250 ± 10	390	1.5×10^6
6	100	1.6 ± 0.1	210 ± 10	390	1.8×10^6
6.5	0.4	1 ± 0.1	180 ± 10	1400	7.6×10^6
6.5	100	1.7 ± 0.1	170 ± 10	360	2.1×10^6
7	0.4	1.00 ± 0.1	180 ± 10	1300	7.4×10^6
7	100	1.8 ± 0.2	150 ± 10	200	1.3×10^6
7.5	0.4	1.1 ± 0.1	80 ± 6	550	6.9×10^6
7.5	100	2.3 ± 0.2			2.5×10^5
8	0.4	1.1 ± 0.1	50 ± 4	260	5×10^6
8	100	3 ± 0.2			2.2×10^4
9	0.4	0.7 ± 0.06			4.1×10^5
9	100	1.1 ± 0.1			1.2×10^5
10	0.4	0.1 ± 0.01			6×10^3
10	100	0.6 ± 0.05			8×10^4

[a] Unless otherwise stated, SD values were below 10% of the mean of four different measurement. In some cases, only the k_{cat}/K_m values were determined from first-order time courses at low substrate concentrations. [b] The [Zn^{2+}]/[enzyme] ratio was determined by ICPMS. The enzyme concentrations were 3 nM and 30 μM for activity and ICPMS measurements, respectively. However, at extreme pH values, the enzyme concentrations were 70 nM (pH 10) and 500 nM (pH 5.0).

of the second site as measured by PIXE and ICPMS on the basis of eq 3 where *n*Zn(II) is the number of zinc ions per molecule of enzyme and [I] the free Zn^{2+} ion concentration.

$$n\mathrm{Zn(II)} = \frac{[\mathrm{I}]}{K_i + [\mathrm{I}]} + 1 \qquad (3)$$

After removal of the excess of metal from the protein samples described in Table 4 by dialysis against metal-free buffer, the Zn content returned to the initial value of one ion per enzyme molecule.

The zinc content of the *Aeromonas hydrophila* metallo-β-lactamase in the absence and presence of 100 μM Zn^{2+} was also strongly pH-dependent (Table 5). In the absence of excess metal and between pH 6.5 and 8, the enzyme contained one zinc ion. The pH dependence of metal binding in the presence of a 100 μM external Zn^{2+} concentration is more complex, but the inhibition at Zn^{2+}/enzyme ratios higher than 1 is clearly visible in the pH range 6.5−9. The addition of increasing NaCl concentrations did not appear to modify the occupancy of both sites (Table 6).

Kinetic Parameters. The K_m and k_{cat} values of the metallo-β-lactamase in the absence and presence of 100 μM Zn^{2+} were determined at different pH values and salt concentrations (Tables 5 and 6). For the mono-Zn enzyme, the K_m value decreased significantly from pH 6 to 8, but since the k_{cat} values also decreased between pH 6.5 and 8, the k_{cat}/K_m values remained maximal in this pH range. A detailed interpretation of the pH effect is made extremely difficult by the interference of the pH-dependent variation of the occupancies of the zinc binding sites. For example, at the extreme pH values (5 and 10), the activity is higher in the presence of 100 μM Zn^{2+}, but in the pH range 6.5−9 the enzyme was consistently more active in the absence of added zinc. Curiously, enzyme with a higher Zn content observed at pH 8 exhibited a very low activity.

Table 6: Metal/Enzyme Molar Ratio ([Zn^{2+}]/[Enzyme) and Kinetic Parameters of the *A. hydrophila* Metallo-β-lactamase at Different Salt Concentrations and [Zn^{2+}] in 15 mM Cacodylate Buffer, pH 6.5[a]

[NaCl] (M)	[Zn^{2+}] in dialysis buffer (μM)	[Zn^{2+}]/[enzyme] ratio[b]	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
0	≤0.4	0.9 ± 0.1	180 ± 10	1300	7.4×10^6
0	100	1.7 ± 0.1	170 ± 10	350	2.07×10^6
0.05	≤0.4	0.9 ± 0.1	180 ± 10	1270	7.2×10^6
0.05	100	1.7 ± 0.1	180 ± 10	340	1.9×10^6
0.1	≤0.4	1 ± 0.1	190 ± 10	1190	6.1×10^6
0.1	100	1.7 ± 0.1	210 ± 10	330	1.6×10^6
0.5	≤0.4	1 ± 0.1	240 ± 10	820	3.4×10^6
0.5	100	1.7 ± 0.15	270 ± 20	170	6.4×10^5
1	≤ 0.4	1.1 ± 0.1	320 ± 20	740	2.3×10^6
1	100	1.7 ± 0.1	330 ± 20	60	1.8×10^5

[a] Unless otherwise stated, SD values were below 10% of the mean of four measurements. [b] Metal/enzyme molar ratios ([Zn^{2+}]/[enzyme]) were determined by ICPMS. For conditions, see the legend of Table 5.

Finally, at pH 6.5, increasing NaCl concentrations decreased the k_{cat}/K_m values by decreasing the k_{cat} and increasing the K_m values, both in the absence and in the presence of 100 μM Zn^{2+}.

Substrate-Induced Inactivation. The three studied penicillins (ampicillin, carbenicillin, and benzylpenicillin) were found to progressively inactivate the enzyme over 20 min time courses. The pseudo-first-order inactivation rate constant for ampicillin (8.17×10^{-3} s^{-1}) was independent of zinc ion concentration up to 100 μM. However, the k_i values for carbenicillin and benzylpenicillin were significantly decreased in the presence of 100 μM Zn^{2+} (6.2×10^{-3} versus 9.3×10^{-3} s^{-1} for carbenicillin, 7.7×10^{-3} versus 13×10^{-3} s^{-1} for benzylpenicillin). This might be related to the more "rigid" (i.e., more stable) structure exhibited by the di- Zn^{2+} enzyme in the thermal and urea-induced denaturation experiments.

The inactivation of the enzyme by ampicillin was also performed at different pH values and salt concentrations in the absence and presence of 100 μM Zn^{2+}. However, the k_i value could only be measured at pH 6.5 in the presence of up to 100 mM NaCl and at pH 7 in the absence and presence of 100 μM Zn^{2+}. No significant variation on the k_i value was detected in these cases. Inactivation was reversible in all cases since the inactivated enzyme regained full activity when diluted in an imipenem solution. Finally, the inactivation was not due to product inhibition, since penicilloic acid (1 mM, a concentration similar to that of penicillin in the time course experiments) had no effects on the initial rate of imipenem hydrolysis over a period of time similar to that used in the experiments where inactivation was observed. These results can be interpreted on the basis of a substrate-induced inactivation mechanism similar to that observed with the active site serine β-lactamase (Citri et al., 1976; Galleni et al., 1988).

Structural Studies of the Apoenzyme and Mono-Zn and Di-Zn Enzymes. The fluorescence emission spectra of the three enzyme forms were similar and exhibited a maximum at 333 nm upon excitation at 280 nm. The intensity slightly but significantly (5%) increased when 1 equiv of zinc was added to the apoenzyme and returned close to the initial value upon saturation of the second site. It seems that the progressive occupation of the first and second binding sites

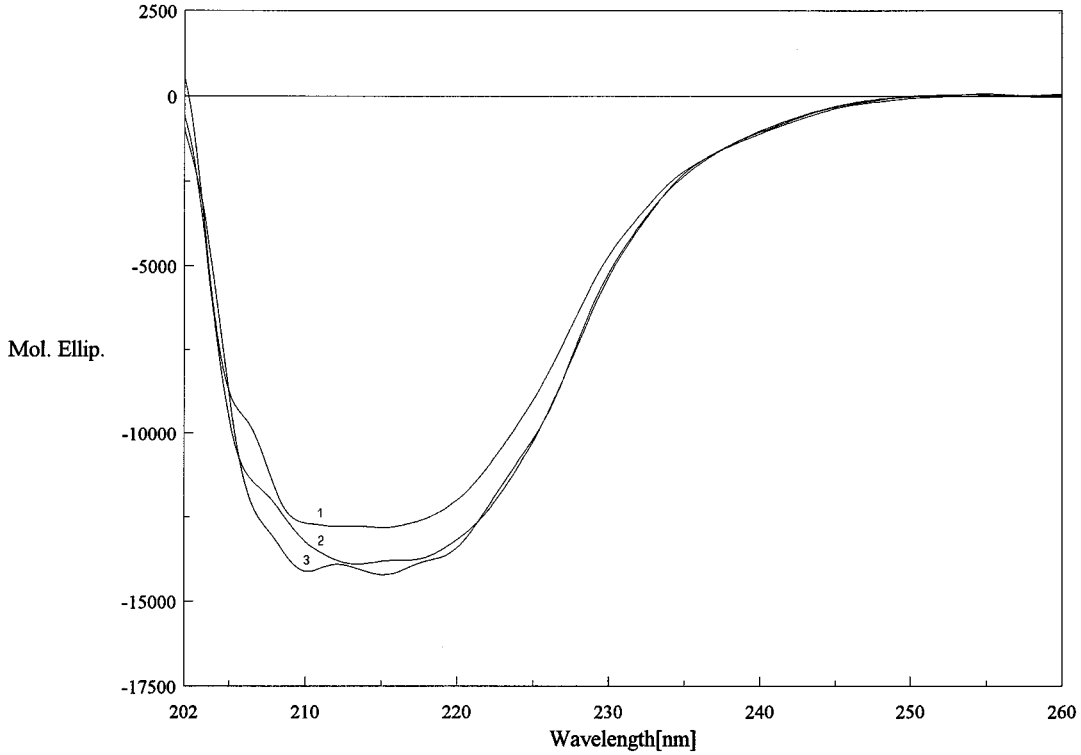


FIGURE 3: Circular dichroism spectra of the apo (1), mono-Zn (2), and di-Zn (3) enzymes (20 μM in all cases).

induces small conformational changes in the protein tertiary structure.

This was confirmed by the far-UV CD spectra of the three forms which also exhibited small but significant differences (Figure 3). However, the helix content did not appear to undergo major variations.

Preliminary measurements also showed that the di-Zn enzyme was more heat-stable than the mono-Zn form, the half-lives at 60 °C being 10 and 5 min, respectively.

More detailed information about the thermal unfolding of the metallo-β-lactamase was obtained from the DSC transitions. Although the thermal denaturation was irreversible in all cases, thus precluding the determination of thermodynamic parameters, the thermograms exhibited a single peak in all cases from which apparent T_m values of 46.2, 60, and 64 °C could be derived for the apo, mono-Zn, and di-Zn forms, respectively (Figure 4). The latter result was obtained in the presence of 1 mM Zn^{2+}. Interestingly, when 0.67 equiv of Zn^{2+} was added to the apoenzyme, two peaks were observed with T_m values of 47 and 58 °C, a result which indicated that rapid Zn exchange did not occur between the apo and the mono-Zn forms (Figure 4). When 1 equiv of Zn^{2+} was added to the apoenzyme, the thermogram was superimposable on that of the native, mono-Zn form. Similar results were obtained for the three different enzyme forms by following the time-dependent decrease of the fluorescence emission at 333 nm in the range of temperatures used in the DSC transitions (data not shown). Again, the thermal stability decreased in the order di-Zn > mono-Zn >> apoenzyme.

The higher stability of the di-Zn enzyme was confirmed with urea as the denaturing agent. The fluorescence emission intensity at 333 nm of the apo, mono-Zn, and di-Zn enzymes decreased after a 16-h incubation in 3, 4, and 5 M urea, respectively (Table 7). After removal of the denaturing agent and when assayed in the presence of 1 equiv of Zn^{2+}, the apo, mono-Zn, and di-Zn forms recovered 50, 50, and 60% of the initial activity, respectively.

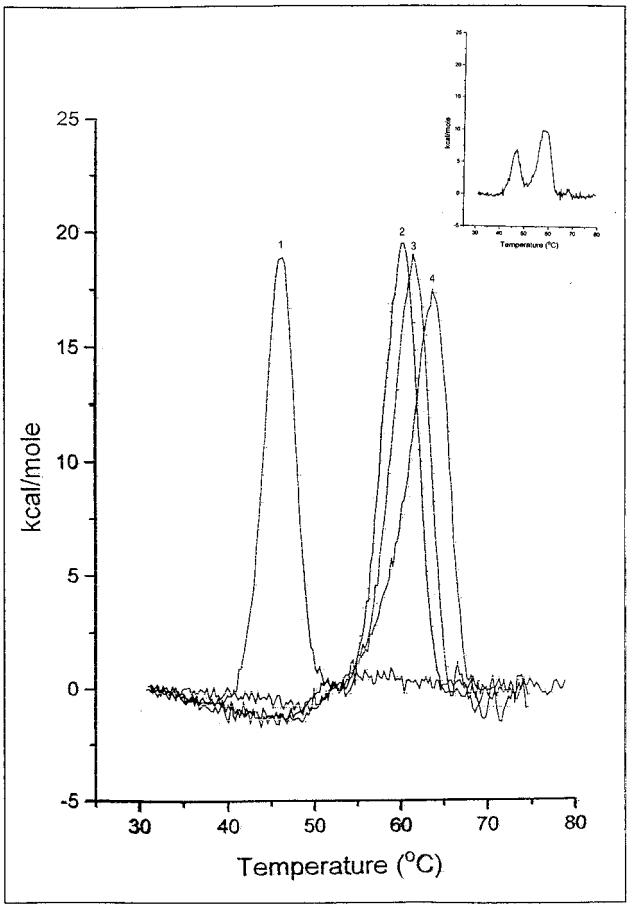


FIGURE 4: DSC transitions of the metallo-β-lactamase (70−111 μM) in the presence of 0 (2), 100 (3), and 1000 μM (4) Zn^{2+} and of the apoenzyme (1). The insert shows the transition profile obtained after addition of 0.67 equiv of Zn^{2+} to the apoenzyme.

Table 7: Relative Fluorescence Emission Intensity at 333 nm of the Different Enzyme Forms after a 16 h Dialysis against Increasing Concentrations of Urea

	relative fluorescence intensity		
[urea] (M)	apoenzyme	mono-Zn enzyme	di-Zn enzyme
0	100	100	100
1	99	98	100
2	99	99	99
3	36	99	99
4	28	47	98
5	28	31	48
6	29	31	33
7	31	33	34
8	32	32	36

[a] Unless otherwise stated, SD values were below 5% of the mean of three different measurements. The enzyme concentration was 2 μM in all cases.

CONCLUSIONS

The *Aeromonas hydrophila* metallo-β-lactamase exhibits maximum activity at pH 6.5 and in the absence of added metal. Under these conditions, one Zn ion is tightly bound per protein molecule which indicates a dissociation constant lower than 20 nM. When the enzyme binds a second Zn ion, a "pure" noncompetitive inhibition is observed with a K_i value of 46 $\pm$ 3 μM. Occupancy of this second binding site does not modify the K_m value but decreases the k_{cat} value to a negligible proportion of that of the mono-Zn enzyme. At higher pH values (pH 7.5−8.0), more metal ions can bind to the enzyme, but it is not clear that this occurs at a well-defined site.

The metal content significantly influences the stability of the protein. The apoenzyme is the most unstable, while the di-Zn form appears to be somewhat more stable than its mono-Zn counterpart, indicating a "tighter" structure, which might be correlated with the slower substrate-induced inactivation observed with the former in the presence of some penicillins. As expected, the enzyme is inactivated by metal chelating agents, but these do not act by scavenging the free metal and therefore transient formation of a ternary enzyme−metal−chelator complex is proposed.

The influence of the second Zn^{2+} ion on enzyme activity is thus very different from that observed in the case of the *Bacteroides fragilis* metallo-β-lactamase, where two Zn^{2+} ions appear to be tightly bound to the protein, resulting in optimal activity.

As a consequence, it is possible that despite a large degree (30%) of sequence similarity, the catalytic mechanisms of the *Aeromonas* and *Bacteroides* enzymes might be somewhat different, but further studies including the determination of the 3D structure of the former enzyme will be necessary to offer a comprehensive explanation to these observations.

The presence of an inhibitory metal binding site in other proteins has been described for several zinc proteases such as carboxypeptidase A (Larsen & Auld, 1989), thermolysin (Holmquist & Vallee, 1974), and angiotensin converting enzyme (Bünning et al., 1989). Interestingly, the inhibitory zinc acts as a competitive inhibitor (Larsen & Auld, 1991) in the case of carboxypeptidase A and as a noncompetitive inhibitor for the metallo-β-lactamase of *A. hydrophila*. A direct interaction between the inhibitory and catalytic zinc is unlikely due to electrostatic repulsive interactions. The structural determinants of the inhibition of carboxypeptidase A by excess zinc were characterized (Gomez-Ortiz et al., 1997). The inhibitory zinc holds a hydroxide and forms a dimetallic monohydroxide moiety analogous to that found in the *B. fragilis* metallo-β-lactamase active sites. A detailed structural study of the *A. hydrophila* metallo-β-lactamase will be necessary in order to understand the chemistry of the interaction between zinc ions and the polypeptide chain and their implication in the regulatory process. The free zinc concentrations in human body fluids and in brackish water (the normal habitat of the *Aeromonas* strains) which can range from nanomolar to micromolar (Jackson, 1989) could affect the β-lacatamase activity.

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