

ENZYMOLOGY:

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Mono- and Binuclear Zn^{2+} - β -Lactamase

ROLE OF THE CONSERVED CYSTEINE IN THE CATALYTIC MECHANISM*

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When expressed by pathogenic bacteria, Zn^{2+} - β -lactamases induce resistance to most β -lactam antibiotics. A possible strategy to fight these bacteria would be a combined therapy with non-toxic inhibitors of Zn^{2+} - β -lactamases together with standard antibiotics. For this purpose, it is important to verify that the inhibitor is effective under all clinical conditions. We have investigated the correlation between the number of zinc ions bound to the Zn^{2+} - β -lactamase from *Bacillus cereus* and hydrolysis of benzylpenicillin and nitrocefin for the wild type and a mutant where cysteine 168 is replaced by alanine. It is shown that both the mono- Zn^{2+} (mononuclear) and di- Zn^{2+} (binuclear) Zn^{2+} - β -lactamases are catalytically active but with different kinetic properties. The mono- Zn^{2+} - β -lactamase requires the conserved cysteine residue for hydrolysis of the β -lactam ring in contrast to the binuclear enzyme where the cysteine residue is not essential. Substrate affinity is not significantly affected by the mutation for the mononuclear enzyme but is decreased for the binuclear enzyme. These results were derived from kinetic studies on two wild types and the mutant enzyme with benzylpenicillin and nitrocefin as substrates. Thus, targeting drug design to modify this residue might represent an efficient strategy, the more so if it also interferes with the formation of the binuclear enzyme.

Zn^{2+} - β -lactamases catalyze the hydrolysis of β -lactam antibiotics by cleaving their β -lactam rings. The production of Zn^{2+} - β -lactamases most often renders bacteria resistant to almost all β -lactam drugs so far designed, including carbapenems. Some of these organisms like *Bacteroides fragilis*, *Serratia marcescens*, *Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa* and *Aeromonas hydrophila* are human pathogens (1), and the search for useful inhibitors for clinical purposes has become of major importance.

The structures of Zn^{2+} - β -lactamases from *Bacillus cereus* strain 569/H/9 and *B. fragilis* have been solved by x-ray crys-

tallography (2–4). Both enzymes contain two metal-binding sites. The zinc ligands are His-86, His-88, and His-149 at the first site (the “three His” site) and those of His-210, Asp-90, and Cys-168 at the second, Cys, site. These residues are highly conserved in almost all the enzymes of the family for which sequence data are available. The first crystal structure of the *B. cereus* enzyme, solved at pH 5.6 and 293 K, showed one zinc ion in the first site (2) but that of the *B. fragilis* enzyme highlighted an oxygen-bridged two-zinc center (3), a result in agreement with the observation that the latter enzyme binds two zinc ions with dissociation constants below 10 μM and reaches its maximum activity when two zinc ions are bound (5). Earlier studies of the *B. cereus* enzyme suggested a much weaker binding of a second equivalent of zinc with marginal effects on the activity (6–7), but further crystallographic studies, performed at 100 K revealed a fully occupied second site (4). The crystallographic data which indicate that Cys-168 is not involved in Zn^{2+} coordination at the high affinity site are apparently in contradiction with spectroscopic studies on the *B. cereus* Co^{2+} and Cd^{2+} derivatives that suggest sulfur ligation at the first site (8).

Despite the different pH conditions used in the crystallographic and biochemical studies, the *B. cereus* and *B. fragilis* enzymes have been hypothesized to be mono- and binuclear Zn^{2+} enzymes, respectively.

The present report investigates this problem for the *B. cereus* Zn^{2+} - β -lactamase and analyzes the catalytic mechanisms of the mono- and binuclear Zn^{2+} enzymes. The results indicate that the conserved Cys-168 is essential for the activity of the mono- Zn^{2+} species but not for the binuclear enzyme. We further present EXAFS¹ data that reconcile the crystallographic and spectroscopic results concerning Zn^{2+} ligation.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—The C168A mutant of the *B. cereus* 569/H/9 Zn^{2+} - β -lactamase was constructed by PCR. Two partially overlapping fragments were amplified using the following primers: 5'GCGT-CCTCGAGAAAGGGTTGATGACATGAA3' (α) plus 5'GATTTCACTAA-AGAGCCTCCAACTAA3'; and 5'AGTTGGAGGCTCTTTAGTGAAT-C3' plus 5'GCGGCTCTAGACGTAATCAACAGATTACAGCAT3' (β). The PCR fragments were gel-purified and combined by overlap PCR in a total volume of 100 μl using 10 ng of each fragment, 100 ng of each oligonucleotide α and β , 2 units of Goldstar polymerase, 1.5 mM MgCl_2 , 200 μM dNTPs, 50 pmol of primer, and 1 ng of *pRTWH012*. The

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¹ The abbreviations used are: EXAFS, extended x-ray absorption fine structure; AAS, atomic absorption spectroscopy; PCR, polymerase chain reaction; WT, wild-type Zn^{2+} - β -lactamase; C168A, mutant of Zn^{2+} - β -lactamase from *B. cereus*, strain 569/H/9 where Cys-168 is replaced by Ala; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol.

corresponding amplimer was digested with *Pst*I and *Cla*I restriction enzymes. The 0.24-kilobase pair fragment was introduced in *pET-BcII* plasmid² to yield *pET-BcIIICA*. Finally, the gene coding for the mature form of the Zn^{2+} - β -lactamase wild-type and C168A mutant were introduced by PCR into the *pTrxFus* plasmid after the gene coding for thioredoxin. Two unique restriction sites (*Kpn*I and *Bam*HI) were introduced before the gene segment coding for the mature form of the β -lactamase and after the STOP codon, respectively. The primers were 5'-CACAAATTTCTCTGTACAGGTACACAAAAGGTAGAGAAAAC3' and 5'-CCCGGGATCCTTAAATATAGTTAGAAGAAAGAGAGAGAA-3'. 25 ng of *pRTWHO12* (for the wild-type) and *pETBcIIICA* (for the C168A mutant) were used as templates. Reaction conditions were 4 min at 95 °C, 30 times (30 s at 95 °C, 1 min at 55 °C, and 1 min at 72 °C). The *Kpn*I-*Bam*HI PCR fragment was cloned into *pTrxFus* in order to create *pCIP32* (wild-type) and *pCIP33* (C168A mutant). The gene was then completely sequenced with the help of an automated laser fluorescent DNA sequencer (Amersham Pharmacia Biotech) to verify that no unwanted mutation had been introduced during the mutagenesis process.

Purification of the Enzymes—The wild-type and C168A mutant from *B. cereus*, strain 569/H/9, was produced by introduction of *pCIP32* and *pCIP33*, respectively, in *Escherichia coli* GI724. The bacteria were grown at 30 °C in 1 liter of induction medium (Invitrogen, San Diego) containing 100 μ g/ml ampicillin as selection agent. At an A_{550} of 0.5, tryptophan (100 μ g/ml final concentration) was added, and the culture was further grown for 120 min. The bacteria were harvested after centrifugation of the culture at $5,000 \times g$ during 15 min. The pellet was resuspended in 100 ml of 10 mM sodium cacodylate buffer, pH 6.5. The cells were broken with the help of a cell disintegrator (Series Z, Constant System, Warwick, UK). After centrifugation at $20,000 \times g$ during 30 min, the supernatant was collected and loaded on a SP Sepharose column (2.5×30 cm, Amersham Pharmacia Biotech, Uppsala, Sweden) pre-equilibrated in 10 mM sodium cacodylate, pH 6.5. The hybrid protein was eluted at a rate of 5 ml/min by a linear salt gradient (0–0.6 M NaCl) in 10 mM sodium cacodylate, pH 6.5. The active fractions were concentrated to 5 ml by ultrafiltration and were dialyzed overnight against 50 mM Tris-HCl, pH 8.0, 1 mM $CaCl_2$, 0.1% Tween 20. Enterokinase (0.1 unit/20 μ g of hybrid protein) was added, and the reaction mixture was incubated at 37 °C for 16 h. The solution was loaded on a MonoS column pre-equilibrated in 10 mM sodium cacodylate, pH 6.5. The β -lactamase was eluted by a linear salt gradient (0–0.5 M) in 10 mM sodium cacodylate, pH 6.5. The active fractions were concentrated to 1 mg/ml as determined by the absorption at 280 nm. The mutant protein was characterized as follows. 1) The mass spectrum, obtained by electrospray mass spectrometry, gave a molar mass of $25,040 \pm 10$ g/mol (the theoretical value is 25,037 g/mol). 2) The N-terminal sequence (6 residues) was identical to that of the WT enzyme. 3) The CD spectra, both in the far and near UV, were superimposable on those of the WT enzyme. 4) The melting temperature, determined according to the modification of the fluorescence spectrum, was identical to that of the WT enzyme (67 ± 0.1 °C) as was the guanidinium chloride concentration resulting in 50% denaturation.

The Zn^{2+} - β -lactamase from *B. cereus* 5/B/6 was produced in *E. coli* MZ1 carrying the *PR2/bls* plasmid as described by Shaw *et al.* (9).

Metal Content Analysis and Preparation of Apoenzymes—To determine the Zn^{2+} content under various conditions, 0.35–0.5-ml samples of the different enzymes at a concentration of either 30 or 50 μ M were dialyzed against 100 ml of the specified buffers containing different concentrations of Zn^{2+} at 4 °C. The protein concentrations were determined after dialysis by measuring the absorbance at 281 nm for the various *B. cereus* enzymes using the following extinction coefficients determined by five different methods including the determination of total amino acid content: 32,700 $M^{-1} cm^{-1}$ for *B. cereus* 5/B/6; 30,500 $M^{-1} cm^{-1}$ for the WT *B. cereus* 569/H/9; and 31,000 $M^{-1} cm^{-1}$ for the *B. cereus* 569/H/9 C168A mutant. The values are accurate to 5% and were used in all protein concentration determinations. Zinc concentrations in samples and in the final dialysis buffers were measured with a Perkin-Elmer 2100 AAS spectrometer in the flame mode or by inductively coupled plasma mass spectroscopy as described by Hernandez Valladares *et al.* (10).

To produce "metal-free" buffers, buffer solutions were purified by extensive stirring with 0.2–0.5% (v/v) of iminodiacetic acid-agarose (Affiland, Liège, Belgium). The residual Zn^{2+} content of the buffers after treatment was approximately 20 nM (10). Standard precautions were taken when the experiments required metal-free conditions (11).

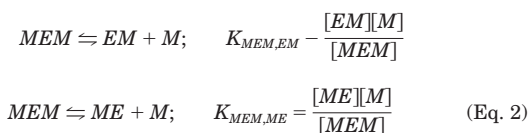
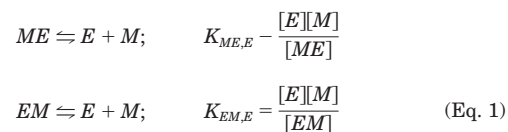
Apoenzymes from strain 5/B/6, 569/H/9 WT, and from the C168A mutant were prepared by dialysis of the corresponding enzymes against 2 changes of 20 mM sodium cacodylate buffer, pH 6.5, containing 1 M NaCl and 20 mM EDTA over 24 h under stirring. EDTA was removed from the resulting apoenzyme solution by 5–7 dialysis steps against the same buffer without metals. In all preparations the remaining zinc content did not exceed 5% as judged by AAS.

Equilibrium Dialysis with ^{65}Zn — ^{65}Zn in 0.1 M HCl (15 mCi/ μ mol) was from Amersham Pharmacia Biotech. Radioactive ^{65}Zn was measured with a Canberra detector model GC1018 connected to a PC via an amplifier and a Canberra ACCUSPEC MCA board.

The dissociation constant relevant for binding of the first zinc ion to the 5/B/6 *B. cereus* enzyme in substoichiometric amounts was determined by dialyzing a 20–400-fold excess of the apoenzyme against radioactive ^{65}Zn . Under these conditions only the mononuclear species of the enzyme can be formed. The samples containing the apoenzyme were placed in a 200- μ l dialysis button (Hampton Research), sealed with a membrane tubing for dialysis and placed in a volume of 2.5 ml of 50 mM HEPES buffer, pH 7.5, in Linbro plate reservoirs for 1–3 days at room temperature under orbital shaking. The final concentration of the enzyme was between 0.15 and 2.75 μ M (with respect to the total volume of dialysis). The samples were dialyzed against 0.36 μ Ci of ^{65}Zn , mixed either with the apoenzyme in the button or in the 2.5-ml reservoir solution.

For studying the binding of zinc in stoichiometric amounts, the 5/B/6 apoenzyme was dialyzed at two different concentrations (6.75 and 14.1 μ M) against various concentrations of isotopically diluted ^{65}Zn .

Equilibrium Model for Zinc Binding—When two sites can bind metals, the following four microscopic equilibria describe metal binding as shown in Equations 1 and 2.



where *M* denotes zinc ions, *E* the enzyme, *EM* the enzyme with zinc bound in the three His site, *ME* the enzyme with zinc bound in the Cys site, and *MEM* the enzyme with zinc ions bound to both sites. In equilibrium dialysis one cannot differentiate between binding to the two sites. Instead macroscopic equilibrium constants are derived. Under substoichiometric (no extra zinc besides ^{65}Zn) and stoichiometric conditions $K_{\text{mono}} = 1/(1/K_{EM,E} + 1/K_{ME,E})$ and $K_{bi} = K_{MEM,EM} + K_{MEM,ME}$ can be determined, respectively. Note that $K_{EM,E} K_{MEM,EM} = K_{ME,E} K_{MEM,ME}$. For a given set of equilibrium constants, the five different equilibrium concentrations $[E]$, $[M]$, $[ME]$, $[EM]$, and $[MEM]$ can be derived by solving the above equations numerically. From these concentrations one can form the ratio of protein-bound Zn^{2+} to total Zn^{2+} (substoichiometric conditions) or protein-bound Zn^{2+} to total protein concentration (stoichiometric conditions). Such calculated ratios were compared with the experimentally determined ratios, and the dissociation constants K_{mono} and K_{bi} were derived by standard nonlinear least squares fitting.

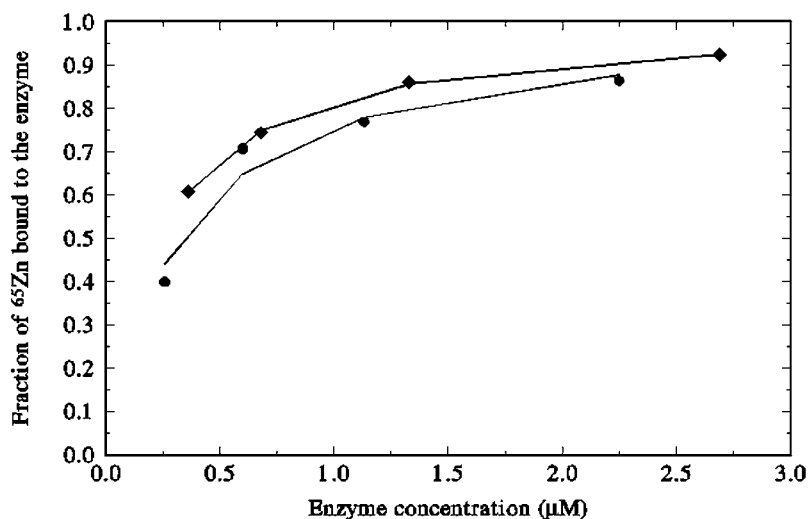
Kinetic Measurements and a Model for the Mechanism—Nitrocefin and benzylpenicillin were from Unipath (Oxford, UK) and Rhone Poulenc (Paris, France), respectively. The hydrolysis of substrates was followed by monitoring the change in absorbance with a Perkin-Elmer Lambda 2 UV/VIS spectrometer at 482 nm for nitrocefin and 235 nm for benzylpenicillin. $K_{m(\text{app})}$ and $k_{\text{cat}(\text{app})}$ values were obtained by the use of initial rates (the complete time courses of hydrolysis were used when the values within the uncertainties were identical to the values obtained with initial rates (12)). The reported $k_{\text{cat}(\text{app})}$ and $K_{m(\text{app})}$ values are the means of at least three single experiments in which the different enzymes were added to the substrate solutions prepared in buffers containing the stated Zn^{2+} concentrations. All experiments were performed at 25 °C in 25 mM HEPES, pH 7.5.

A steady state model in which $k_{\text{cat}(\text{app})}$ and $K_{m(\text{app})}$ contains contributions from both the mononuclear and binuclear Zn^{2+} enzyme via $k_{\text{cat},1}$, $k_{\text{cat},2}$, $K_{m,1}$ and $K_{m,2}$ where the subscripts 1 and 2 refer to the mono- and binuclear Zn^{2+} enzymes, respectively, is presented in Equations 3 and 4.

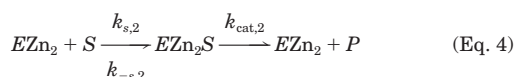
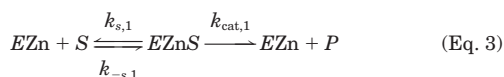
Steady State Model for the Mono and Binuclear Zn^{2+} - β -Lactamase—

² M. Galleni, unpublished observations.

FIG. 1. Binding of radioactive ^{65}Zn to apo- β -lactamase from *B. cereus* strain 5/B/6. From equilibrium dialysis of the 5/B/6 enzyme against ^{65}Zn (no extra Zn^{2+} added) in 50 mM HEPES, pH 7.5, without (●) and with 1 M NaCl (◆). The standard deviation on the experimental points are below 10%. The curves were obtained by standard nonlinear least squares fitting using the equilibrium model given under "Experimental Procedures."



The kinetic data were analyzed according to the following steady state model involving catalysis by both the mono- and the binuclear Zn^{2+} enzymes as follows.



where $E\text{Zn}^{2+}$, $E\text{Zn}_2^{2+}$, $E\text{Zn}^{2+}S$, and $E\text{Zn}_2^{2+}S$ are the mononuclear and binuclear enzyme without and with bound substrate, respectively. In addition the binding of the second Zn^{2+} ion to both $E\text{Zn}^{2+}$ and $E\text{Zn}_2^{2+}S$ is assumed to be in rapid equilibrium as shown in Equations 5 and 6.



and corresponding macroscopic dissociation constants are K_{bi} and K'_{bi} , respectively. We now use the steady state assumptions that $d[E\text{Zn}^{2+}S]/dt$ and $d[E\text{Zn}_2^{2+}S]/dt$ are 0. Also we refer to the initial conditions where $[S] = [S_0]$ and $[P] = 0$. On this basis, Equations 7–11 can be derived as follows.

$$\nu = k_{\text{cat},1}[E\text{Zn}S] + k_{\text{cat},2}[E\text{Zn}_2S] \quad (\text{Eq. 7})$$

$$k_{s,1}[E\text{Zn}][S_0] + k_{s,2}[E\text{Zn}_2][S_0] = (k_{-s,1} + k_{\text{cat},1})[E\text{Zn}S] + (k_{-s,2} + k_{\text{cat},2})[E\text{Zn}_2S] \quad (\text{Eq. 8})$$

$$[E_0] = [E\text{Zn}] + [E\text{Zn}_2] + [E\text{Zn}S] + [E\text{Zn}_2S] \quad (\text{Eq. 9})$$

$$K_{\text{bi}} = [E\text{Zn}][\text{Zn}]/[E\text{Zn}_2] \quad (\text{Eq. 10})$$

$$K'_{\text{bi}} = [E\text{Zn}S][\text{Zn}]/[E\text{Zn}_2S] \quad (\text{Eq. 11})$$

where ν is the steady state velocity. The k_{cat} and K_m values for the mono- and binuclear zinc enzyme are $k_{\text{cat},1}$ and $k_{\text{cat},2}$ and $K_{m,1}$ and $K_{m,2}$, respectively. Here $K_{m,1} = (k_{-s,1} + k_{\text{cat},1})/k_{s,1}$ and $K_{m,2} = (k_{-s,2} + k_{\text{cat},2})/k_{s,2}$. Solving Equations 7–11 yields ν/E_0 , $k_{\text{cat}(\text{app})}$, and $K_{m(\text{app})}$.

$$\frac{\nu}{[E_0]} = \frac{k_{\text{cat}(\text{app})}[S_0]}{K_{m(\text{app})} + [S_0]} \quad (\text{Eq. 12})$$

where

$$k_{\text{cat}(\text{app})} = \frac{k_{\text{cat},1}K'_{\text{bi}} + k_{\text{cat},2}[\text{Zn}]}{K_{\text{bi}} + [\text{Zn}]} \quad (\text{Eq. 13})$$

and

$$K_{m(\text{app})} = \frac{\left(K_{m,1}K'_{\text{bi}} + \frac{k_{s,2}}{k_{s,1}}K_{m,2}[\text{Zn}]\right)(K_{\text{bi}} + [\text{Zn}])}{\left(K_{\text{bi}} + \frac{k_{s,2}}{k_{s,1}}[\text{Zn}]\right)(K'_{\text{bi}} + [\text{Zn}])} \quad (\text{Eq. 14})$$

If $k_{s,2}/k_{s,1}$ is equal to 1 the equation for $K_{m(\text{app})}$ simplifies, and furthermore, a simple linear relation between $K_{m(\text{app})}$ and $k_{\text{cat}(\text{app})}$ can be derived by elimination of $[\text{Zn}^{2+}]$ in Equations 13 and 14 for $K_{m(\text{app})}$ and $k_{\text{cat}(\text{app})}$. We thus get

$$K_{m(\text{app})} = \frac{K_{m,1}K'_{\text{bi}} + K_{m,2}[\text{Zn}]}{K'_{\text{bi}} + [\text{Zn}]} \quad (\text{Eq. 15})$$

$$K_{m(\text{app})} = \frac{K_{m,1}k_{\text{cat},2} - K_{m,2}k_{\text{cat},1} + (K_{m,2} - K_{m,1})k_{\text{cat}(\text{app})}}{k_{\text{cat},2} - k_{\text{cat},1}} \quad (\text{Eq. 16})$$

As Equation 16, used for deriving the values for $K_{m,1}$ and $K_{m,2}$ (Table III), assumes that $k_{s,2}/k_{s,1} = 1$, it is important to know how critical this restriction is concerning the actual values derived. For instance by choosing the following values ($k_{s,1} = 0.5 \mu\text{M}^{-1} \text{s}^{-1}$; $k_{-s,1} = 200 \text{s}^{-1}$; $k_{s,2} = 0.5 \mu\text{M}^{-1} \text{s}^{-1}$; $k_{-s,2} = 825 \text{s}^{-1}$) for the hydrolysis of benzylpenicillin by the mutant, one calculates the values for $K_{m,1}$ and $K_{m,2}$ given in Table III. Increasing the $k_{s,2}$ value while keeping $K_{m,2} [(k_{-s,2} + k_{\text{cat},2})/k_{s,2}]$ constant did not result in significant modifications. By contrast, progressively decreasing the same value also keeping $K_{m,2} [(k_{-s,2} + k_{\text{cat},2})/k_{s,2}]$ constant resulted in increasing deviations from linearity. At this point, it should be noted that an equilibrium model where $K_{m,1} = k_{-s,1}/k_{s,1}$ and $K_{m,2} = k_{-s,2}/k_{s,2}$ (thus implying $k_{\text{cat},1} < k_{-s,1}$ and $k_{\text{cat},2} < k_{-s,2}$) yields an equation identical to Equation 16 without any further assumption. It is clear that increasing $k_{s,2}$ brings the steady state model closer to the equilibrium situation, whereas a decrease of the same constant increases the differences between the two models and can result in significant deviations from linearity in the $K_{m(\text{app})}$ versus $k_{\text{cat}(\text{app})}$ plot. Standard nonlinear least squares fittings were applied in fitting the kinetic data to the model.

EXAFS Spectroscopy—The EXAFS studies were performed with the enzyme produced by strain 5/B/6. The sample was prepared by dialysis of the native Zn^{2+} enzyme against two changes of 25 mM Bis-Tris buffer, pH 6.5, containing 1 M ammonium acetate and 10 μM Zn^{2+} followed by an additional dialysis against the same buffer without Zn^{2+} . The presence of a high ionic strength was necessary to avoid precipitation of the highly concentrated enzyme. After centrifugation the enzyme concentration was $710 \pm 35 \mu\text{M}$. The $[\text{Zn}^{2+}]/[E]$ ratio was 1.2 ± 0.1 as determined by AAS. The EXAFS data were collected at beamline D2 at the European Molecular Biology Laboratory Outstation Hamburg, and samples were measured as frozen solutions at 18 K in fluorescence mode (13). The energy resolution was better than 2.5 eV. The data were analyzed using the computer program packages EXPROG (14) and EXCURV92 (developed by N. Binsted, S. W. Campbell, S. J. Gurman, and P. Stephenson at Daresbury Laboratory, United Kingdom).

RESULTS

Zn^{2+} Binding to *B. cereus* Zn^{2+} - β -Lactamase—After equilibrium dialysis against 25 mM HEPES, pH 7.5, containing 1 M NaCl and 15 μM Zn^{2+} , the $[\text{Zn}^{2+}]/[E]$ ratios ($[E] = 0.175 \mu\text{M}$) obtained for the 5/B/6 and 569/H/9 *B. cereus* enzymes as determined by AAS were 2.0 ± 0.1 , in both cases implying a K_{bi} value lower than 10 μM .

Analysis of data (Fig. 1) from equilibrium dialysis of the 5/B/6 enzyme against ^{65}Zn (no extra Zn^{2+} added) gave the values for the equilibrium constants K_{mono} shown in Table I. Stoichiometric binding of Zn^{2+} (^{65}Zn) to the metal-free 5/B/6 *B. cereus* β -lactamase was also studied in equilibrium dialysis experiments with 14.1 μM apoenzyme against 10, 20, 40, and 80 μM Zn^{2+} (no NaCl added) and with 6.75 μM apoenzyme (1 M NaCl) against 20, 40, and 80 μM Zn^{2+} . For the fitting of these data, K_{mono} was constrained to the value obtained under substoichiometric conditions. The results are shown in Table I. The dissociation constants K_{mono} and K_{bi} do not depend on the presence of NaCl within the experimental error and K_{bi} is about 10 times larger than K_{mono} .

For comparison with the crystallization conditions used by Carfi *et al.* (2), the Zn^{2+} content of the enzyme from strain 5/B/6 was determined by AAS after dialyzing 0.35 ml of 50 μM apoenzyme against 100 ml of 25 mM citrate buffer, pH 5.6, containing 1 M NaCl. The $[Zn^{2+}]/[E]$ ratio was <0.1 without added Zn^{2+} ; 0.6 and 0.8 with 13.3 and 62.5 μM external Zn^{2+} , respectively. As the $[Zn^{2+}]/[E]$ ratio was less than 1 even at 62.5 μM Zn^{2+} K_{bi} was ignored in analyzing the data. If a second zinc ion binds it does so very weakly. The fitted value of K_{mono} is given in Table I.

Correlation between Zinc Concentration and Hydrolysis of Benzylpenicillin and Nitrocefin by *B. cereus* Zn^{2+} - β -Lactamases—Table II shows the kinetic parameters obtained for the 5/B/6 and 569/H/9 enzymes at different Zn^{2+} concentrations. For the two WT enzymes the K_m values do not significantly vary with the Zn^{2+} concentration. Note that measurements in metal-depleted buffer (20 nM $[Zn^{2+}]$ or less) with final enzyme concentrations far below the dissociation constant K_{mono} results in $k_{cat(app)}$ values not significantly different from zero with benzylpenicillin as substrate in contrast to the results obtained with nitrocefin where the 5/B/6 enzyme exhibits full

activity in metal-depleted buffer, even in the presence of 10 μM EDTA in the assay buffer. For benzylpenicillin this could simply be explained by the release of Zn^{2+} (fast on the kinetic time scale) and for nitrocefin by a strong increase in affinity for Zn^{2+} upon binding of nitrocefin most likely by a decrease of the rate constant for the release of Zn^{2+} . Additional evidence comes from the observation that the apoenzyme at a concentration of 36 nM is partly reactivated in the presence of nitrocefin prepared in metal-depleted buffer (20 nM $[Zn^{2+}]$ or less). For the 5/B/6 enzyme with nitrocefin as substrate, k_{cat} also appears to be independent of the Zn^{2+} concentration (above 20 nM, Table II). Thus, binding of a second Zn^{2+} ion, if it occurs, has, in this case, no influence on the kinetic parameters. That only one Zn^{2+} ion is necessary for full activity was confirmed by titrating the apoenzyme with increasing zinc concentration. The result demonstrates a virtual linear dependence upon zinc concentration up to the apoenzyme concentration of 10 μM followed by a plateau (Fig. 2 (Δ)). Fig. 2 also demonstrates that the specific activity *versus* Zn^{2+} concentration for benzylpenicillin changes essentially in the submicromolar range of Zn^{2+} , a result reflecting the formation of the mononuclear enzyme. Thus, as with nitrocefin, the mononuclear enzyme is likely to be fully active. No significant difference was observed if the reaction was started by adding the apoenzyme to the reaction mixture. The specific activity for the hydrolysis of benzylpenicillin for the 569/H/9 WT enzyme is shown in Fig. 3. Again as with the 5/B/6 enzyme, the major changes in specific activity occur at submicromolar values of Zn^{2+} concentrations. However, both the specific activity and the $k_{cat(app)}$ values further increase above 1 μM Zn^{2+} . Kinetic parameters fitted using the model developed under "Experimental Procedures" also highlight a $k_{cat,2}$ value about twice as high as $k_{cat,1}$ (Table III). For the 569/H/9 WT enzyme with 150 μM nitrocefin as substrate, similar rates with residual and 1 μM Zn^{2+} were observed but increased 2-fold upon further addition of Zn^{2+} (Fig. 3). As the values of $K_{m(app)}$ are about 20 times lower than 150 μM , the specific activity supplies a very good approximation of $k_{cat(app)}$. Therefore the specific activity shown in Fig. 3 was fitted to Equation 13 for $k_{cat(app)}$ given under "Experimental Procedures." The results are given in Table III. The specific activity of the 569/H/9 enzyme *versus* 150 μM nitrocefin in citrate buffer, pH 5.6, containing 100 μM Zn^{2+} is 46% that in HEPES buffer, pH 7.5, also containing 100 μM Zn^{2+} demonstrating that at this pH only the mononuclear enzyme is active.

Correlation between Zinc Concentration and Hydrolysis for

TABLE I
Equilibrium constants from equilibrium dialysis in 25 mM HEPES, pH 7.5

Enzyme	NaCl	K_{mono}	K_{bi}
	M	μM	μM
WT 5/B/6	0	0.33 ± 0.06	3.3 ± 1.0
WT 5/B/6	1	0.23 ± 0.01	4 ± 2
WT 5/B/6 ^a	1	10 ± 3	
C168A 569/H/9	0	0.4 ± 0.2	10 ± 3

^a Dialyzed against 1 M NaCl, 25 mM citrate buffer, pH 5.6.

TABLE II
 Zn^{2+} dependence of the kinetic parameters obtained with the *B. cereus* enzymes in 25 mM HEPES buffer, pH 7.5, at 25 °C
The enzyme concentrations in the assay were in the 10–50 nM range. SD values were below 10% of the mean of three measurements.

[Zn]	5/B/6			569/H/9 WT		
	$k_{cat(app)}$	$K_{m(app)}$	$k_{cat(app)}/K_{m(app)}$	$k_{cat(app)}$	$K_{m(app)}$	$k_{cat(app)}/K_{m(app)}$
μM	s^{-1}	μM	$M^{-1} s^{-1}$	s^{-1}	μM	$M^{-1} s^{-1}$
Nitrocefin						
≤ 0.02	44	67	6.6×10^5	8.7	5.9	1.5×10^6
1	38	59	6.4×10^5	9.2	5.5	1.7×10^6
10	38	68	5.6×10^5	11	4.7	2.3×10^6
100	32	56	5.7×10^5	18	5.7	3.1×10^6
1000	35	50	7.0×10^5	21	6.5	3.2×10^6
Benzylpenicillin						
≤ 0.02	<10		$<10^3$	<10		$<10^3$
1	349	890	3.9×10^5	165	323	5.1×10^5
5	462	1070	4.3×10^5	218	400	5.5×10^5
10	448	1030	4.3×10^5	253	452	5.6×10^5
20	448	1025	4.4×10^5	271	398	6.8×10^5
30	432	970	4.5×10^5	289	413	7.0×10^5
50	448	1010	4.4×10^5	287	431	6.7×10^5
100	487	1115	4.4×10^5	320	440	7.3×10^5

FIG. 2. **Interaction between the 5/B/6 enzyme and zinc ions.** Δ , activity recovery of the 5/B/6 apoenzyme upon titration with Zn^{2+} . The 5/B/6 apoenzyme was diluted to $10 \mu\text{M}$ in the assay buffer containing from 0 to $30 \mu\text{M}$ Zn^{2+} . The activity of each sample was determined by measuring initial rate of hydrolysis of $150 \mu\text{M}$ nitrocefin prepared in metal-free buffer. The final enzyme concentration was 36 nM . $\blacksquare$, influence of Zn^{2+} concentration on the specific activity of Zn^{2+} - β -lactamase from strain 5/B/6 with 1 mM benzylpenicillin as substrate. The final enzyme concentrations were 40 – 70 nM . The assay buffer was 25 mM HEPES, pH 7.5 , in both cases. In both cases the standard deviation on the experimental points are below 5% .

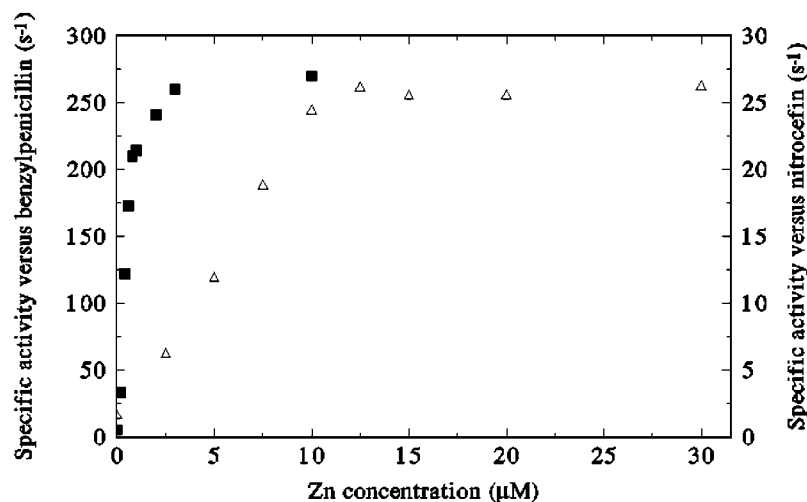
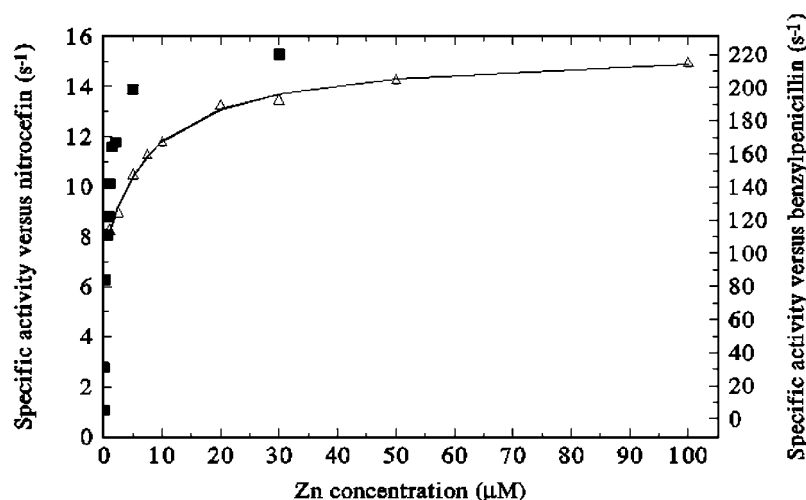


FIG. 3. **Interaction between the 569/H/9 enzyme and zinc ions.** Δ , influence of Zn^{2+} concentration on the hydrolysis of $150 \mu\text{M}$ nitrocefin by the 569/H/9 enzyme. The assay buffer contained different Zn^{2+} concentrations. The experimental data shown were fitted as described in the text (line). The final enzyme concentration was 24 nM . $\blacksquare$, influence of Zn^{2+} concentration on the specific activity of Zn^{2+} - β -lactamase from strain 569/H/9 with 1 mM benzylpenicillin as substrate. The final enzyme concentrations were 40 – 70 nM . The assay buffer was 25 mM HEPES, pH 7.5 . In both cases the standard deviation on the experimental points are below 5% .

TABLE III
Derived kinetic parameters from the model presented under "Experimental Procedures"

Substrate	Enzyme	$k_{\text{cat},1}$	$k_{\text{cat},2}$	$K_{m,1}$	$K_{m,2}$	K'_{bi}
		s^{-1}		μM	μM	μM
Nitrocefin	WT 569/H/9	7.3 ± 0.4^a	15.6 ± 0.3^a	6.0 ± 0.6^b	6.0 ± 0.6^b	8.2 ± 1.7^a
Nitrocefin	C168A	0.4 ± 0.1^c	$\geq 80^d$	10 ± 1^c		
Benzylpenicillin	WT 569/H/9	136 ± 18^a	307 ± 12^a	330 ± 70^e	440 ± 90^e	5 ± 2^a
Benzylpenicillin	C168A	1.8 ± 0.2^c	194 ± 18^a	405 ± 50^e	2040 ± 50^e	38 ± 8^a

^a Value obtained by fitting the data from Table II or from Fig. 4 (C168A) for benzylpenicillin using Equations 12 and 13 as shown under "Experimental Procedures." Note that for the mutant $K'_{\text{bi}} = 10 \mu\text{M}$ (Table I).

^b Estimate from Table II.

^c Measured using a 1:1 ratio of $[\text{Zn}]/[\text{E}]$. This gave $K_{m,1} = 520 \pm 52$ for the mutant with benzylpenicillin.

^d $k_{\text{cat}(\text{app})}$ at the maximal Zn concentration shown in Fig. 5.

^e Value obtained by fitting the data shown in Fig. 6 using Equation 16 under "Experimental Procedures" and K'_{bi} from the fit using Equation 13.

the C168A Mutant—After equilibrium dialysis of the C168A mutant ($0.1 \mu\text{M}$) against 25 mM HEPES, pH 7.5 , containing 0.6 , 5.0 , and $10 \mu\text{M}$ Zn^{2+} , the $[\text{Zn}^{2+}]/[\text{E}]$ ratios determined by inductively coupled plasma mass spectroscopy were 0.7 , 1.2 , and 1.6 , respectively. Thus, it is also possible to bind two zinc ions to the mutant. The derived equilibrium constants are given in Table I.

The kinetic properties of the C168A mutant are completely different from that of the WT enzyme. The activity in the presence of residual Zn^{2+} is negligible for both substrates and both the $k_{\text{cat}(\text{app})}$ and $K_{m(\text{app})}$ values for benzylpenicillin increase with increasing Zn^{2+} concentration (Fig. 4). The kinetic parameters for the mononuclear species for the C168A mutant were derived by mixing $2 \mu\text{M}$ apoenzyme with solutions con-

taining $1.9 \mu\text{M}$ Zn^{2+} and different concentrations of benzylpenicillin and nitrocefin. The results are given in Table III. When fitting the values of $k_{\text{cat}(\text{app})}$ for benzylpenicillin (Fig. 4) to the equation for $k_{\text{cat}(\text{app})}$, $k_{\text{cat},1}$ was fixed to 1.8 s^{-1} (Table III). At Zn^{2+} concentrations above $1 \mu\text{M}$, the specific activity versus nitrocefin also increased (Fig. 5), and the shape of the curve was close to that of the $k_{\text{cat}(\text{app})}$ for benzylpenicillin. In Fig. 6 the values of $K_{m(\text{app})}$ are plotted versus $k_{\text{cat}(\text{app})}$ (from Table II for the 569/H/9 enzyme and from Fig. 4 for the mutant). Fig. 6 demonstrates a linear dependence of $K_{m(\text{app})}$ versus $k_{\text{cat}(\text{app})}$, and corresponding least squares fitting to straight lines gave the values for $K_{m,1}$ and $K_{m,2}$ presented in Table II.

EXAFS Spectroscopy—The rigid structure of systems like imidazole is well known. Therefore, in EXAFS restrained re-

FIG. 4. Influence of Zn^{2+} concentration on the catalytic parameters. $K_m(\text{app})$ (■) and $k_{\text{cat}}(\text{app})$ (△) of the C168A mutant from strain 569/H/9 with benzylpenicillin as substrate. The enzyme concentration varied from about 1.5 μM for the low Zn^{2+} concentrations to 0.15 μM for the high Zn^{2+} concentrations. The values for $k_{\text{cat}}(\text{app})$ were fitted as described in the text (line). The standard deviations on the experimental points are below 10%.

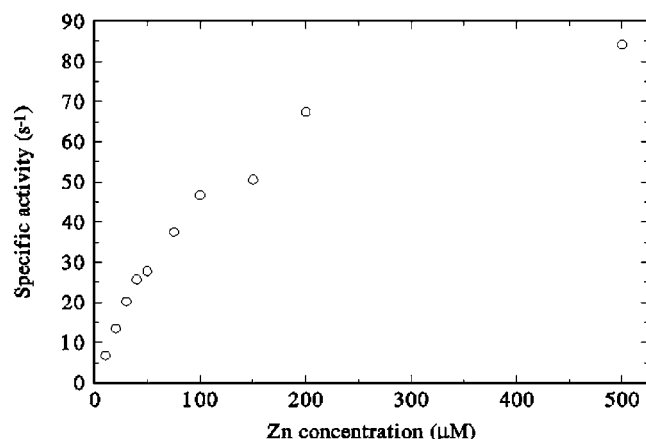
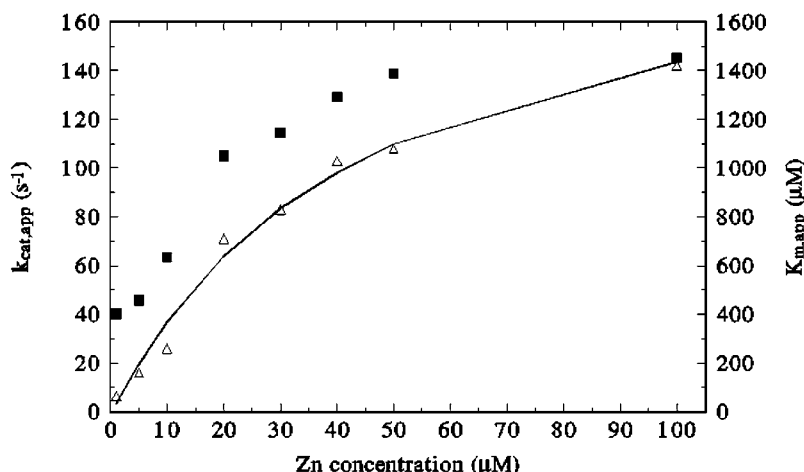


FIG. 5. Influence of Zn^{2+} concentration on the hydrolysis of 100 μM nitrocefin by the C168A mutant. The assay buffer (see legend to Fig. 2) contained different Zn^{2+} concentrations. The final enzyme concentration varied from 9–53 nM. The standard deviations on the experimental points are below 5%.

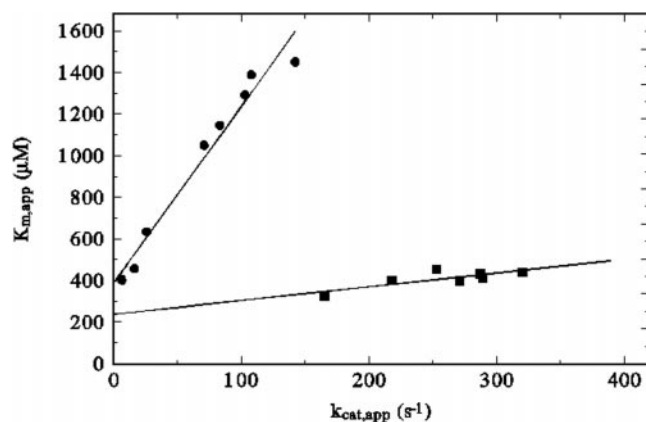


FIG. 6. Plot of the data in Table II and Fig. 4 as $K_m(\text{app})$ versus $k_{\text{cat}}(\text{app})$. The circles are for the mutant and the boxes for the WT. The lines were from fits as described in the text.

finement is applied to such problems (15). We first modeled the coordination sphere of zinc with three histidine residues and a water molecule as ligands as would be expected from the crystal structure data if zinc is not coordinated to the site with cysteine as a ligand. The interpretation of the extracted k^2 weighted fine structure by these assumptions result in *fit 1* shown in Fig. 7, where the corresponding Fourier transform clearly indicates a missing contribution at about 2.25 Å. This can be accounted for by the contribution of a cysteine ligand

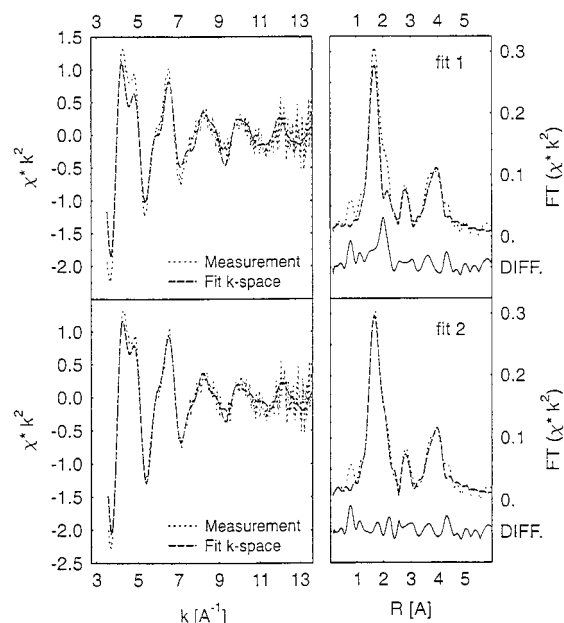


FIG. 7. The effect of including a sulfur atom in a fit to the EXAFS spectrum of the *B. cereus* 5/B/6 enzyme at pH 6.5 and with 1.2 zinc ions per enzyme molecule. To the left is the EXAFS spectrum with the corresponding fits and to the right the corresponding Fourier transforms. *Fit 1* is without a contribution from sulfur and *fit 2* with a contribution of 0.5 sulfur. The plots of the differences between the Fourier transform of experiment and fit are indicated by "DIFF."

(16). A fractional contribution of sulfur was also observed in EXAFS spectroscopy on the *B. cereus*, 569/H/9, enzyme at pH 6.0 also containing about 1 eq of Zn^{2+} (17). However, the authors did not give any interpretation of the presence of this sulfur. From the amplitude it was obvious that, on average, less than one sulfur atom was present. To obtain an upper limit for the number of sulfur ligands, the corresponding Debye-Waller parameters were fixed, because of their strong correlation with coordination numbers in EXAFS spectra. The Debye-Waller parameter of the sulfur atom accounts only for dynamic disorder and the static disorder between all the enzyme units, whereas the Debye-Waller parameter for the nitrogen also bound to the central Zn^{2+} additionally accounts for the static disorder within this unit (between the three imidazole ligands). Thus the Debye-Waller parameter for sulfur should be much smaller than for nitrogen. To estimate the upper limit for the presence of sulfur atoms, it was fixed to an even slightly lower value (0.003 Å²). Analyzing the data with this model resulted in a significant improvement of the fit and a maximum coordina-

tion number for sulfur of 0.5 (Fig. 7). The corresponding parameters given in Table IV show that the improvement of the fit is only due to this sulfur contribution, because all other parameters were identical within their errors. The difference between the Fourier transforms of experiment and theory clearly indicated the absence of any further contribution above the noise level. The structures derived from x-ray diffraction data show that the Cys-168 residue is not close enough to coordinate the zinc at the 3-histidine site. As the EXAFS data show a fractional zinc coordination by sulfur, the only solution is a partial occupancy of the second site with cysteine, aspartate, and histidine as zinc ligands in the mononuclear species. However, because the Zn^{2+} /enzyme ratio was 1.2 ± 0.1 in the present case, part of the sulfur signal could also arise from a weakly occupied binuclear zinc enzyme.

DISCUSSION

Existence of a Mono- and a Binuclear Zinc Enzyme with Different Kinetic Properties—Equilibrium dialysis in citrate buffer, pH 5.6, provided an estimation of K_{mono} of 10 μM for the 5/B/6 enzyme and no evidence for binding of a second Zn^{2+} ion. The fact that the activity of the 569/H/9 enzyme at pH 5.6 versus nitrocefin is only about 50% that observed in HEPES buffer, pH 7.5 (when both contain 100 μM Zn^{2+}), correlates well with the absence of a second enzyme-bound Zn^{2+} ion. The preferential occupancy of the three-His site in the mononuclear species revealed in the crystal structure (Fig. 8) (2) is then

TABLE IV
Theoretical fits with and without sulfur to the EXAFS spectrum of the 5/B/6 *B. cereus* Zn - β -lactamase at pH 6.5 having 1.2 Zn ions per enzyme molecule

All parameters with given error margins were adjusted in the refinement.

Ligand	Fit 1			Fit 2		
	N_i	R_i [Å]	$2\sigma^2$ [Å ²]	N_i	R_i [Å]	$2\sigma^2$ [Å ²]
His	3			3		
N		1.98 (1)	0.004 (1)		1.99 (1)	0.004 (1)
C		2.99 (1)	0.015 (2) ^a		2.99 (1)	0.014 (4) ^a
C		3.05 (1)	0.015 (2) ^a		3.07 (1)	0.014 (4) ^a
N		4.13 (1)	0.008 (2) ^b		4.14 (2)	0.008 (2) ^b
C		4.19 (1)	0.008 (2) ^b		4.21 (1)	0.008 (2) ^b
H ₂ O	1			1		
O		3.27 (4)	0.015 (2) ^a		3.25 (5)	0.014 (4) ^a
Cys	0.54 (5)			0		
S		2.27 (1)	0.003			

^a The Debye-Waller factors for atoms with similar distances were assumed to be identical.

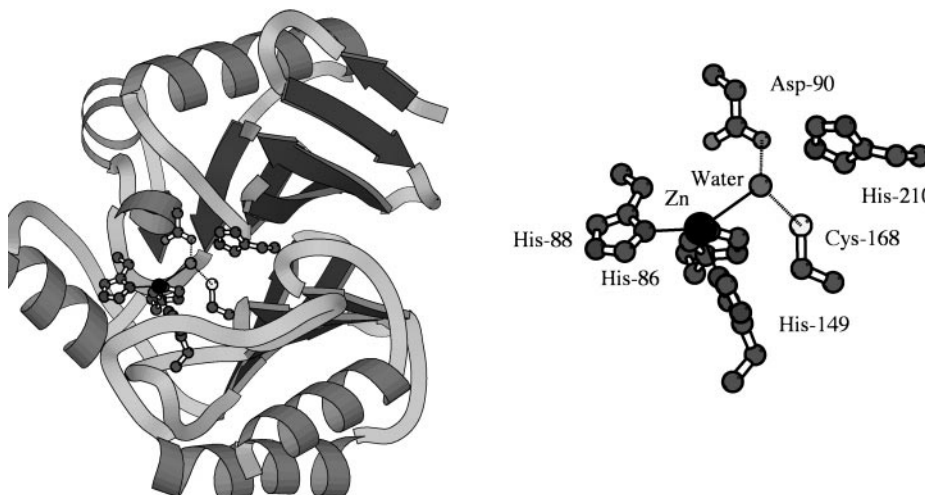
^b The Debye-Waller factors for atoms with similar distances were assumed to be identical.

satisfactorily explained by the much weaker binding of Zn^{2+} to the Cys site at pH 5.6. However, already at pH 6.5 the EXAFS data indicate a significant occupancy of the Cys site in the so-called mononuclear species. This together with the observation of the binding of a second zinc at pH 7.5 with a weaker binding (Table I) is consistent with a dominant population of the three-His site together with a relatively lower population of the Cys site at pH values higher than or equal to 6.5 and at stoichiometries close to or below 1. The mononuclear zinc enzyme thus corresponds to a protein with only 1 zinc ion per molecule which could be either in the three-His site or the Cys site. Dialyzing the enzymes against a large concentration of zinc always results in the formation of a binuclear enzyme. In agreement with this, recent crystallographic studies of the 569/H/9 enzyme at pH 7.5 show a fully occupied second site.³

The k_{cat} value of the 569/H/9 enzyme increases 2-fold upon binding of the second Zn^{2+} ion for both substrates (Table II, see also Fig. 3). From this alone, it is not possible to assign different mechanisms for the mononuclear and the binuclear zinc enzymes because half activity for an average of one zinc ion bound per protein molecule could equally well be explained by the coexistence of enzyme molecules with no zinc ions or enzyme molecules with two zinc ions. However, the data for the 5/B/6 enzyme changes this for two reasons. First, there is no increase in the k_{cat} value with increasing zinc concentrations for nitrocefin (Table II). Second, the activity recovery curve with nitrocefin starting with the 5/B/6 apoenzyme shows unambiguously that maximum activity is obtained with only one zinc ion bound, *i.e.* no further increase in activity occurs upon formation of the binuclear enzyme (Fig. 2, see also Table II). This is further confirmed by the full activity of the 5/B/6 enzyme with the same substrate when no extra zinc is added (Table II). It is obvious that upon binding to the enzyme, nitrocefin (but not benzylpenicillin) increases the affinity for the first zinc ion as demonstrated by the activity with no extra zinc added (Table II). With benzylpenicillin as substrate and the 5/B/6 enzyme, the data are also consistent with a 2-fold rise in k_{cat} from the mononuclear zinc enzyme to the binuclear enzyme (Table II, see also Fig. 2) as for the 569/H/9 enzyme with both substrates. The conclusion is then that the kinetic properties of the mononuclear and the binuclear enzymes can differ according to the substrate and the enzyme. In a recent work (18) a similar conclusion was drawn for the Zn^{2+} - β -lactamase from *B. fragilis*. Despite the differences observed in the kinetic parameters between the mono- and binuclear spe-

³ R. Paul-Soto and J. Wouters, unpublished results.

FIG. 8. Structure of the active site of the 569/H/9 β -lactamase at pH 5.6. The drawing is produced by Molscript (P. J. Kraulis) using the Brookhaven Protein Data Bank pdb1bmc file.



cies, a large proportion of activity persists for the mononuclear enzyme. Thus the formation of a binuclear enzyme is not necessary for efficient catalysis.

Note that the 5/B/6 and 569/H/9 enzymes differ only by 17 substitutions. Although the Thr-173 $\rightarrow$ Ala and Ala-175 $\rightarrow$ Ser substitutions in the 5/B/6 enzyme relative to the 569/H/9 enzyme are not far from the active site, they fail to explain, at the present time, the different values of the kinetic parameters as well as the different dependences on zinc concentrations of the two WT enzymes.

The Role of the Conserved Cysteine Residue—The kinetic analysis of the 569/H/9 WT enzyme and its C168A mutant shows that the affinity for benzylpenicillin is identical for the WT and the mutant for the mononuclear but different for the binuclear Zn^{2+} enzyme, whereas hydrolysis of benzylpenicillin is strongly reduced for the mono- Zn^{2+} mutant relative to its wild-type counterpart but not for the binuclear Zn^{2+} species. With nitrocefin, it is also clear that the mutant activity is higher than that of the WT at high Zn^{2+} concentrations (compare Figs. 3 and 5). This suggests that the mononuclear and binuclear Zn^{2+} enzyme function via different mechanisms. Indeed, Cys-168 is essential for efficient hydrolysis by the mononuclear enzyme but not by the binuclear species.

As suggested by Concha *et al.* (3) the two zinc ions could be bridged by a shared hydroxyl which would attack the carbonyl carbon of the β -lactam ring, but the exact role of the Cys residue in the mononuclear enzyme remains to be elucidated.

Nevertheless, the crucial importance of Cys-168 in catalysis by the mononuclear Zn^{2+} enzyme and its suggested irrelevance for hydrolysis by the binuclear enzyme is supported by the fact that the C168A mutant is able to bind two Zn^{2+} ions at pH 7.5 with dissociation constants below 10 μM . Cys-168 is thus not essential for binding of the second zinc ion. Interestingly the *Pseudomonas maltophilia* enzyme where the otherwise conserved cysteine residue is a serine residue also does bind two Zn^{2+} ions but the third ligand of the second zinc ion is now a His side chain situated in a completely different part of the polypeptide chain (His-89) (19). The possible formation of the binuclear Zn^{2+} enzyme may represent a kind of sophistication in an alternative mechanism that does not require Cys-168.

The present work shows that the catalytic mechanism of Zn^{2+} enzymes requiring one metal ion for activity may become somewhat more efficient by acquisition of co-catalytic sites with two zinc ions in close proximity acting as a unit center (20). However, as shown by studies performed with the *B. fragilis* enzyme (18, 21), the catalytic efficiency of the binuclear enzyme is only marginally superior to that of its mononuclear counterpart with some substrates and is even lower with other ones (18).

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