Mono- and Binuclear Zn\(^{2+}\)-\(\beta\)-Lactamase

ROLE OF THE CONSERVED CYSTEINE IN THE CATALYTIC MECHANISM

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Raquel Paul-Soto‡§, Rogert Bauer‡, Jean-Marie Frère‡, Moreno Galleni‡, Wolfram Meyer-Klaucke‡, Hans Nolting‡, Gian Maria Rossolini**‡, Dominique de Seny§, Maria Hernandez-Valladares‡, Michael Zeppenauer‡, and Hans-Werner Adolph‡ ‡‡

From ‡‡Fachrichtung 12.4 Biochemie, Universität des Saarlandes, D-66041 Saarbrücken, Germany, §Centre d’Ingénierie des Protéines, Institut de Chimie B6, Université de Liège, Sart-Tilman, B-4000 Liège, Belgium, †EMBL-Outstation Hamburg at DESY, Notkestrasse 85, D-22603 Hamburg, Germany, ‡Department of Physics, The Royal Veterinary and Agricultural University, Dk-1871 Frederiksberg C, Denmark, and the **Dipartimento di Biologia Molecolare, Sezione di Microbiologia, Università di Siena, Siena 53100, Italy

When expressed by pathogenic bacteria, Zn\(^{2+}\)-\(\beta\)-lactamases induce resistance to most \(\beta\)-lactam antibiotics. A possible strategy to fight these bacteria would be a combined therapy with non-toxic inhibitors of Zn\(^{2+}\)-\(\beta\)-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\(^{2+}\)-\(\beta\)-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\(^{2+}\) (mono-nuclear) and di-Zn\(^{2+}\) (binuclear) Zn\(^{2+}\)-\(\beta\)-lactamases are catalytically active but with different kinetic properties. The mono-Zn\(^{2+}\)-\(\beta\)-lactamase requires the conserved cysteine residue for hydrolysis of the \(\beta\)-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\(^{2+}\)-\(\beta\)-lactamases catalyze the hydrolysis of \(\beta\)-lactam antibiotics by cleaving their \(\beta\)-lactam rings. The production of Zn\(^{2+}\)-\(\beta\)-lactamases most often renders bacteria resistant to all \(\beta\)-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\(^{2+}\)-\(\beta\)-lactamases from Bacillus cereus strain 569/H/9 and B. fragilis have been solved by x-ray crystallography (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 \(\mu\)M 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\(^{2+}\)-\(\beta\)-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 EXAFS\(^{1}\) 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\(^{2+}\)-\(\beta\)-lactamase was constructed by PCR. Two partially overlapping fragments were amplified using the following primers: 5’ GCCGT-CCTCGAGAAGGGTTGATGACATGAA3’ (a) plus 5’ GATTTCACATAGGACGGCTTCAACTAA3’ plus 5’ AGTGGAGGCTTCTTATTGAGAACAT-T C3’ plus 5’ GCCGGCTCTAGCTAATCCAGAAGGTGATTACTCCATGAA3’ (β). The PCR fragments were gel-purified and combined by overlap PCR in a total volume of 100 \(\mu\)l using 10 ng of each fragment, 100 ng of each oligonucleotide α and β, and 2 units of Goldstar polymerase. 1.5 mm MgCl\(_2\), 200 \(\mu\)M 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\(^{2+}\)-\(\beta\)-lactamase from B. cereus, strain 569/H/9 where Cys-168 is replaced by Ala; Bis-Tris, 2-[bis(2-hydroxyethyl)aminol]-2-(hydroxyethyl)-propane-1,3-diol.
corresponding amplier was digested with PstI and ClaI restriction enzymes. The 0.24-kilobase pair fragment was introduced in pET-BclII plasmid to yield pET-BclICA. Finally, the gene coding for the mature form of the Zn\(^{2+}\)-lactamase wild-type and C168A mutant were introduced by PCR into the pTRxfus plasmid after the gene coding for thiocyanate 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 µg/ml ampicillin as selection agent. At an A\(_{600}\) of 0.5, tryptophan (0.1 µg/ml as determined by the absorption at 280 nm) was added, and the culture was further grown for 120 min. The bacteria were harvested after centrifugation of the culture at 5,000 × 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 × 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 mg/ml as determined by the absorption at 280 nm) 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 molecular mass (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\(^{2+}\)-lactamase from *B. cereus* 5/B/6 was produced in *E. coli* M1Z1 carrying the PR2/bla 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 µM were dialyzed against 10 mM 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 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 PerkinElmer 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 mM NaCl and 20 mM EDTA over 24 h under stirring. EDTA was removed from the resulting apoapozyme solution by 5–7 dialysis steps against the same buffer and EDTA. In all preparations the remaining zinc content did not exceed 5% as judged by AAS.

**Equilibrium Dialysis with 65Zn\(^{2+}\)-Zn\(^{2+}\)**—For studying the zinc in stoichiometric amounts, the 5/B/6 apoapozyme was dialyzed at two different concentrations (6.75 and 14.1 µM) against various concentrations of isotopically diluted 65Zn.

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

\[
\begin{align*}
\text{ME} & \rightleftharpoons E + M; \\
K_{\text{MEM}E} & = \frac{[EM]}{[E][M]} \\
\text{EM} & \rightleftharpoons E + M; \\
K_{\text{MEM}EM} & = \frac{[EMM]}{[E][M][EM]} \\
\text{MEM} & \rightleftharpoons ME + M; \\
K_{\text{MEM}ME} & = \frac{[MEM]}{[E][M][EM]} \\
\text{MEM} & \rightleftharpoons ME + M; \\
K_{\text{MEM}EM} & = \frac{[MEM]}{[E][M][EM]} \\
\end{align*}
\]

where M denotes zinc ions, E the enzyme, EM 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 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 65Zn) and stoichiometric conditions \(K_{\text{ENZO}} = 1/(1/K_{\text{MEM}EM} + 1/K_{\text{MEM}ME})\) and \(K_{\text{MEM}EM} = K_{\text{MEM}ME} = K_{\text{MEM}EM} \pm K_{\text{MEM}ME} \) Can be determined, respectively. Note that \(K_{\text{MEM}EM} \neq K_{\text{MEM}ME} \pm K_{\text{MEM}EM} \). For a given set of equilibrium constants, the five different equilibrium concentrations \([E],[M],[EM],[EMS],[MEM] \) 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 \(K_{\text{MEM}}\) and \(K_{\text{ME}}\) 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 492 nm for nitrocefin and 235 nm for benzylpenicillin. \(k_{\text{cat(app)}}\) and \(k_{\text{cat(app)}}\) 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 \(k_{\text{cat(app)}}\) and \(k_{\text{cat(app)}}\) values are the means of at least three single experiments in which the different buffers were added to the experiments 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 \(k_{\text{cat(app)}}\) and \(k_{\text{cat(app)}}\) contains contributions from both the mononuclear and binuclear Zn\(^{2+}\) enzyme via \(k_{\text{cat(app)}}\) and \(k_{\text{cat(app)}}\) 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\(^{2+}\)-Lactamase**—

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2 M. Galleni, unpublished observations.
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.

\[
\begin{align*}
E_{Zn} + S & \xrightarrow{k_{s,1}} E_{ZnS} \xrightarrow{k_{cat,1}} E_{Zn} + P \quad (\text{Eq. 3}) \\
E_{Zn} + S & \xrightarrow{k_{s,2}} E_{ZnS} \xrightarrow{k_{cat,2}} E_{Zn2} + P \quad (\text{Eq. 4})
\end{align*}
\]

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

\[
E_{Zn} + Zn \equiv E_{Zn}^{5+} \quad (\text{Eq. 5})
\]

\[
E_{ZnS}^{2+} + Zn \equiv E_{ZnS}^{5+} \quad (\text{Eq. 6})
\]

and corresponding macroscopic dissociation constants are \(K_{s,1}\) and \(K_{s,2}\), respectively. We now use the steady state assumptions that \(d[E_{Zn}^{2+}] / dt\) and \(d[E_{Zn}^{2+}] / dt\) are zero. 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.

\[
\nu = k_{cat,1}[E_{ZnS}] + k_{cat,2}[E_{ZnS}] \quad (\text{Eq. 7})
\]

\[
k_{s,1}[E_{ZnS}] + k_{s,2}[E_{ZnS}] = (k_{s,1} + k_{cat,1})[E_{ZnS}] + (k_{s,2} + k_{cat,2})[E_{ZnS}] \quad (\text{Eq. 8})
\]

\[
[E_{0}] = [E_{Zn}] + [E_{ZnS}] + [E_{ZnS}] \quad (\text{Eq. 9})
\]

\[
K_{s,1} - [E_{Zn}][Zn]/[E_{ZnS}] \quad (\text{Eq. 10})
\]

\[
K_{s,2} = [E_{ZnS}][Zn]/[E_{ZnS}] \quad (\text{Eq. 11})
\]

where \(\nu\) is the steady state velocity. The \(k_{cat}\) and \(K_{s}\) values for the mono- and binuclear zinc enzyme are \(k_{cat,1}\) and \(k_{cat,2}\) and \(K_{s,1}\) and \(K_{s,2}\), respectively. Here \(K_{s,1} = (k_{s,1} + k_{cat,1})/k_{cat,1}\) and \(K_{s,2} = (k_{s,2} + k_{cat,2})/k_{cat,2}\). Solving Equations 7–11 yields to \(E_{0}\), \(k_{cat,1}\) and \(k_{cat,2}\) for \(K_{s}\) and \(K_{cat}\) for the mono- and binuclear zinc enzyme,

\[
\nu = \frac{k_{cat,app}[S]}{[E_{0}] + k_{cat,app}[S]} \quad (\text{Eq. 12})
\]

where

\[
k_{cat,app} = \frac{k_{cat,1}K_{s,1} + k_{cat,2}K_{s,2}}{K_{s,1} + [Zn]} \quad (\text{Eq. 13})
\]

and

\[
K_{cat,app} = \frac{(K_{s,1}K_{s,1} + k_{s,1}K_{s,2})[Zn]}{(K_{s,1} + k_{s,1}K_{s,2})[Zn]} \quad (\text{Eq. 14})
\]

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

\[
K_{cat,app} = \frac{k_{cat,1}K_{s,1} + k_{cat,2}K_{s,2}[Zn]}{K_{s,1} + [Zn]} \quad (\text{Eq. 15})
\]

\[
K_{cat,app} = \frac{k_{cat,1}K_{s,1} - K_{s,1}K_{cat,1} + (K_{s,2} - K_{cat,2})[Zn]_{cat,app}}{K_{s,1} - K_{cat,1}} \quad (\text{Eq. 16})
\]

As Equation 16, used for deriving the values for \(K_{cat,1}\) and \(K_{cat,2}\) (Table III), assumes that \(k_{cat,1}/k_{cat,2} = 1\), it is important to know how critical this restriction is concerning the actual values derived. For instance by choosing the following values: \(k_{cat,1} = 0.5 \mu\text{M}^{-1}\text{s}^{-1}\), \(k_{cat,2} = 200 \mu\text{M}^{-1}\text{s}^{-1}\), \(k_{cat,1} = 0.5 \mu\text{M}^{-1}\text{s}^{-1}\), \(k_{cat,2} = 200 \mu\text{M}^{-1}\text{s}^{-1}\), \(k_{cat,1} = 0.5 \mu\text{M}^{-1}\text{s}^{-1}\), and \(k_{cat,2} = 200 \mu\text{M}^{-1}\text{s}^{-1}\) for the hydrolysis of benzylpenicillin by the mutant, one calculates the values for \(K_{cat,1}\) and \(K_{cat,2}\) given in Table III. Increasing the \(k_{cat,2}\) value while keeping \(K_{cat,1}\) constant did not result in significant modifications. By contrast, progressively decreasing the same value also keeping \(K_{cat,1}\) constant resulted in increasing deviations from linearity. At this point, it should be noted that an equilibrium model where \(K_{cat,1} = k_{s,1}/k_{cat,1}\) and \(K_{cat,2} = k_{s,2}/k_{cat,2}\) (thus implying \(k_{cat,1}/k_{cat,2}\) and \(k_{cat,2}/k_{cat,1}\) yields an equation identical to Equation 16 without any further assumption. It is clear that increasing \(k_{cat,2}\) 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 \(K_{cat,app}\) versus \(k_{cat,app}\) 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 \(\mu\text{M}\) 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 \([\text{Zn}^{2+}] / [\text{E}]\) ratio was 1.2 \(\pm\) 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 program computer packages EXPROG (14) and EXCURV92 (developed by N. Binsted, S. W. Cambell, S. J. Gurman, and P. Stephenson at Daresbury Laboratory, United Kingdom).

**RESULTS**

Zn\(^{2+}\) Binding to B. cereus Zn\(^{2+}\)-\(\beta\)-Lactamase—After equilibrium dialysis against 25 mM HEPES, pH 7.5, containing 1 \(\mu\text{M}\) NaCl and 15 \(\mu\text{M}\) Zn\(^{2+}\), the \([\text{Zn}^{2+}] / [\text{E}]\) ratios ([E]) were 0.175 \(\mu\text{M}\) obtained for the 5/B/6 and 569/119 B. cereus enzymes as determined by AAS were 2.0 \(\pm\) 0.1, in both cases implying a \(K_{bi}\) value lower than 10 \(\mu\text{M}\).
Analysis of data (Fig. 1) from equilibrium dialysis of the 5B/6 enzyme against $^{65}\text{Zn}$ (no extra $\text{Zn}^{2+}$ added) gave the values for the equilibrium constants $K_{\text{mono}}$ shown in Table I. Stoichiometric binding of $\text{Zn}^{2+}$ ($^{65}\text{Zn}$) to the metal-free 5B/6 B. cereus $\beta$-lactamase was also studied in equilibrium dialysis experiments with 14.1 $\mu\text{M}$ apoenzyme against 10, 20, 40, and 80 $\mu\text{M}$ $\text{Zn}^{2+}$ (no NaCl added) and with 6.75 $\mu\text{M}$ apoenzyme (1 $\text{mM}$ NaCl) against 20, 40, and 80 $\mu\text{M}$ $\text{Zn}^{2+}$. For the fitting of these data, $K_{\text{mono}}$ was constrained to the value obtained under substoichiometric conditions. The results are shown in Table I. The dissociation constants $K_{\text{mono}}$ and $K_{\text{bi}}$ do not depend on the presence of NaCl within the experimental error and $K_{\text{bi}}$ is about 10 times larger than $K_{\text{mono}}$.

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

**Correlation between Zinc Concentration and Hydrolysis of Benzylpenicillin and Nitrocefin by B. cereus $\text{Zn}^{2+}$-$\beta$-Lactamases**—Table II shows the kinetic parameters obtained for the two WT enzymes at different $\text{Zn}^{2+}$ concentrations. Note that measurements in metal-depleted buffer (20 nM $\text{Zn}^{2+}$) or less with final enzyme concentrations far below the dissociation constant. Table II shows the kinetic parameters obtained for the two WT enzymes at different $\text{Zn}^{2+}$ concentrations.
the C168A Mutant—After equilibrium dialysis of the C168A mutant (0.1 mM) against 25 mM HEPES, pH 7.5, containing 0.6, 5.0, and 10 mM Zn$^{2+}$, the [Zn$^{2+}$]/[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 $k_{\text{cat(app)}}$ and $K_{\text{cat(app)}}$ 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 mM apoenzyme with solutions containing 1.9 mM Zn$^{2+}$ and different concentrations of benzylpenicillin and nitrocefin. The results are given in Table III. When fitting the values of $k_{\text{cat(app)}}$ for benzylpenicillin (Fig. 4) to the equation for $k_{\text{cat(app)}}$, $k_{\text{cat,1}}$ was fixed to 1.8 s$^{-1}$ (Table III). At Zn$^{2+}$ concentrations above 1 mM, the specific activity versus nitrocefin also increased (Fig. 5), and the shape of the curve was close to that of the $k_{\text{cat(app)}}$ for benzylpenicillin. In Fig. 6 the values of $K_{\text{m(app)}}$ are plotted versus $k_{\text{cat(app)}}$ (from Table II for the 569/H/9 enzyme and from Fig. 4 for the mutant). Fig. 6 demonstrates a linear dependence of $K_{\text{m(app)}}$ versus $k_{\text{cat(app)}}$ and corresponding least squares fitting to straight lines gave the values for $K_{\text{m,1}}$ and $K_{\text{m,2}}$ presented in Table II.

**EXAFS Spectroscopy**—The rigid structure of systems like imidazole is well known. Therefore, in EXAFS restrained re-
Refinement 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 (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 Deby-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 Å$^2$). 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 \(K_{\text{mono}}\) of 10 \(\mu\)M 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 \(\mu\)M 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 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 \(k_{\text{cat}}\) 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 \(k_{\text{cat}}\) 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 \(k_{\text{cat}}\) 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\(^{2+}/\)\(\beta\)-lactamase from *B. fragilis*. Despite the differences observed in the kinetic parameters between the mono- and binuclear spe-

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\(\text{TABLE IV}
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Theoretical fits with and without sulfur to the EXAFS spectrum of the 5/B/6 *B. cereus* Zn\(^{2+}\)-\(\beta\)-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 | \(N_i\) | \(R_i\) \([\text{\AA}]\) | \(2\sigma^2\) \([\text{\AA}^2]\) | \(N_i\) | \(R_i\) \([\text{\AA}]\) | \(2\sigma^2\) \([\text{\AA}^2]\) |
|--------|--------|--------|--------|--------|--------|--------|
| His    | 3      | 1.96 (1) | 0.004 (1) | 3      | 1.99 (1) | 0.004 (1) |
| N      | 2.99 (1) | 0.015 (2) | 2.99 (1) | 0.014 (4) |
| C      | 3.05 (1) | 0.015 (2) | 3.07 (1) | 0.014 (4) |
| N      | 4.13 (1) | 0.008 (2) | 4.14 (2) | 0.008 (2) |
| C      | 4.19 (1) | 0.008 (2) | 4.21 (1) | 0.008 (2) |
| H\(_2\)O | 1      | 3.27 (4) | 0.015 (2) | 3.25 (5) | 0.014 (4) |
| O      | 0.54 (5) | 0.003 |
| 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.

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\(^3\) R. Paul-Soto and J. Wouters, unpublished results.

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**Fig. 8.** Structure of the active site of the 569/H/9 \(\beta\)-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 → Ala and Ala-175 → 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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