Preference of Cd(II) and Zn(II) for the Two Metal Sites in *Bacillus cereus* β-Lactamase II: A Perturbed Angular Correlation of γ-rays Spectroscopic Study†

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ABSTRACT: Cd-substituted forms of the *Bacillus cereus* metallo-β-lactamases (BCII) were studied by perturbed angular correlation of γ-rays (PAC) spectroscopy. At very low [Cd]:[apo-β-lactamase] ratios, two nuclear quadrupole interactions (NQI) were detected. For [Cd]:[apo-β-lactamase] ratios between 0.8 and 3.0, two *new* NQIs appear, and the spectra show that up to 2 cadmium ions can be bound per molecule of apoenzyme. These results show the existence of two interacting Cd-binding sites in BCII. The relative populations of the two NQIs found at low [Cd]:[apo-β-lactamase] ratios yielded a 1:3 ratio for the microscopic dissociation constants of the two different metal sites (when only one cadmium ion is bound). X-ray diffraction data at pH 7.5 demonstrate that also for Zn(II) two binding sites exist, which may be bridged by a solvent molecule. The measured NQIs could be assigned to the site with three histidines as metal ligands (three-His site) and to the site with histidine, cysteine, and aspartic acid as metal ligands (Cys site), respectively, by PAC measurements on the Cys168Ala mutant enzyme. This assignment shows that cadmium ions preferentially bind to the Cys site. This is in contrast to the preference of Zn(II) in the hybrid Zn(II)Cd(II) enzyme, where an analysis of the corresponding PAC spectrum showed that Cd(II) occupied the Cys site, whereby Zn(II) occupied the site with three histidines. The difference between Zn(II) and Cd(II) in affinity for the two sites is combined with the kinetics of hydrolysis of nitrocefin for different metal ion substitutions (Zn_2E, ZnE, Cd_2E, CdE, and ZnCdE) to study the function of the two metal ion binding sites.

The first crystal structure of a metallo-β-lactamase was obtained for the enzyme from *Bacillus cereus*, strain 569/H/9, so far the most-studied Zn-β-lactamase. The structure at 2.5 Å resolution was obtained at pH 5.7 (*1*) and shows a zinc ion coordinated to three His residues (86, 88, and 149) and a water molecule. Whereas the structure confirmed early ^{1}H NMR studies (*2*, *3*), it was in disagreement with suggestion of Cys ligation from spectroscopic studies on the Co and Cd derivatives (*4*). With a distance of 4.4 Å the conserved Cys168 residue cannot coordinate to the zinc ion. In a structure obtained at 100 K and refined to 1.8 Å a second metal ion binding site was found to be partially occupied by Zn(II) (*5*). The second zinc ion is located 3.7 Å from the first and is coordinated to His-210, Asp-90, and Cys-168 as the protein ligands. The Zn-β-lactamase from *Bacteroides fragilis* crystallized at pH 7.5 in the presence of 10 μM Zn(II) revealed a binuclear metal center with a solvent molecule bridging two zinc ions, which are coordinated to the same set of conserved residues as found for the *B. cereus* enzyme at 100 K (*6*, *7*). A similar structure was obtained in the presence of cadmium ions instead of zinc ions (*8*). However, the structure of the *B. fragilis* Zn-β-lactamase obtained at pH 9.0 seemed to lack a bridging water molecule between the two zinc ions (*9*), but a reinvestigation showed that also at this pH a water molecule could be bridging the zinc ions (Duée et al., manuscript in preparation; PDB file 2bmi). The picture evolved by the different crystallographic studies is thus confusing with respect to metal site interactions and stoichiometry of metal ion binding. In particular, the role of the two metal sites in catalysis still needs to be clarified.

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[1] Abbreviations: PAC, perturbed angular correlation of γ-rays; NQI, nuclear quadrupole interaction; IDA, iminodiacetic acid; BCII, β-lactamase from *Bacillus cereus*; Mrad/s, 10^6 radians per second; AAS, atomic absorption spectroscopy; CdE, β-lactamase with one cadmium ion bound per enzyme molecule; Cd_2E, β-lactamase with two cadmium ions bound per enzyme molecule (and correspondingly for ZnE and Zn_2E); ZnCdE, β-lactamase with one zinc and one cadmium ion bound per enzyme molecule.

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In the present study we focus on the metal ion coordination geometries present in the *B. cereus* Zn-β-lactamase in solution, using perturbed angular correlation of γ-rays (PAC) spectroscopy on Cd(II) derivatives. The results from PAC spectroscopy are combined with studies of kinetics for hydrolysis of nitrocefin with different metal ion substitutions, to elucidate the function of the metal ion binding site(s). Furthermore, X-ray diffraction data were obtained on the native enzyme at 100 K at pH 7.5.

MATERIALS AND METHODS

The overexpression and purification of the enzymes were described by Paul-Soto et al. (*10*). Apoenzymes of the 5/B/6 and 569/H/9 wild-type enzymes and the C168A mutant of the latter were also prepared as described in Paul-Soto et al. (*10*). Carrier-free ^{109}Cd in 0.08 M HCl (89.1 mCi/μmol) was from Amersham. Nitrocefin was from Unipath (Oxford, England). Buffer solutions and dialysis membranes were metal-depleted as described in Paul-Soto et al. (*10*). Atomic absorption spectroscopy (AAS) measurements were performed with a Perkin-Elmer 2100 atomic absorption spectrometer in the flame mode.

Determination of Kinetic Parameters. The hydrolysis of nitrocefin was followed by monitoring the increase of absorbance at 482 nm, resulting from the opening of the β-lactam ring, with a Perkin-Elmer Lambda 2 UV/VIS spectrometer. K_m and k_{cat} values were obtained by analysis of the initial rates of hydrolysis at eight different substrate concentrations. The reported values are the means of at least three single determinations. K_m and k_{cat} values were obtained by fitting the experimental initial rate values to the Henri−Michaelis equation by a standard nonlinear least squares computer program. All experiments were performed at 25 °C in 25 mM HEPES, pH 7.5.

Equilibrium Model for Cd(II) Binding. Binding of metal ions at two different metal sites can be completely characterized by the microscopic dissociation constants $K_{A,1}$, $K_{B,1}$, $K_{A,2}$, and $K_{B,2}$, where A and B represents the two different metal sites. A,1 denotes one metal ion bound to site A and A,2 denotes both metal sites occupied by binding a metal ion to site B with site A already occupied. Only three of the four constants are independent. PAC spectroscopy can in principle determine all the microscopic constants whereas equilibrium dialysis only determines the macroscopic constants $1/K_{mono} = 1/K_{A,1} + 1/K_{B,1}$ and $K_{bi} = K_{A,2} + K_{B,2}$.

With stoichiometric amounts of Cd(II) relative to apoenzyme, the two coordination geometries in the binuclear species can be monitored. Note that in this case the NQIs accounting for the two binuclear coordination geometries should be equally populated in the PAC spectrum.

Equilibrium Dialysis Experiments. For estimating the binding constants of a second equivalent of Cd to the different enzyme species, dialysis experiments were performed at 40 °C with the corresponding apoenzymes. To prevent zinc contamination, the apoenzymes were diluted to 50 μM with buffer containing 500 μM EDTA, and the 0.5 mL samples were first dialyzed against 100 mL of buffer also containing 500 μM EDTA followed by three dialysis steps against iminodiacetic acid- (IDA-) treated buffer containing different Cd concentrations. The final Zn(II) content of the enzyme was always less than 12% as verified by AAS. The mixed Zn(II), Cd(II) derivative was obtained by dialyzing 0.5 mL samples of 50 μM native 5/B/6 Zn-β-lactamase twice against 100 mL of 25 mM HEPES buffer, pH 7.5, containing concentrations of Cd^{2+} ranging from 10 to 100 μM.

K_{mono} and K_{bi} for Cd(II) binding were determined by equilibrium dialysis against radioactive ^{109}Cd as previously described for Zn(II) (*10*). The dissociation constant K_{mono} for binding of 1 equiv of Cd to the 5/B/6 *B. cereus* enzyme was obtained by dialysis of excess apoenzyme against 0.26 μCi of carrier-free ^{109}Cd (substoichiometric conditions). Under these conditions it is very unlikely that two metal ions simultaneously occupy the same enzyme molecule, and only the formula for K_{mono} was used to analyze the experimental data. To determine the dissociation constant for binding of a second equivalent of Cd^{2+}, K_{bi}, the apoenzyme was dialyzed against an excess of Cd ions (1.24−10.4 μCi/μmol, obtained by isotopic dilution of 26 μCi of ^{109}Cd with nonradioactive Cd). The numerical integration program CHEMSIM was used for evaluation of the dissociation constants. This program allows analysis of binding experiments by use of combined equilibria (*11*). For the fitting of the data with stoichiometric amounts of Cd(II), K_{mono} was constrained to the value obtained under substoichiometric conditions.

Preparation of PAC Samples. The ^{111m}Cd was produced by the Cyclotron Department at the University Hospital in Copenhagen. Preparation and purification of ^{111m}Cd were described previously (*12*). Solutions (10−40 μL) containing about 10−40 pmol of ^{111m}Cd in H_2O were mixed with a volume from 40 to 100 μL of metal-depleted HEPES, MES, or Tris buffers containing either no additional metal ions or various stoichiometric amounts of either cadmium or zinc ions or both. To these solutions various amounts of apoenzyme were added. The pH was adjusted at room temperature by addition of small amounts of 1−5 M HCl or NaOH. A 10 min waiting period allowing the metal(s) to bind was included at room temperature before finally sucrose was added to give a 54% w/w solution. Increasing the period for cadmium ions to bind to 20 min did not change the corresponding PAC spectrum. Within 10 min the sample was cooled to 1 °C. The sample volumes ranged from 200 to 1000 μL. All PAC experiments were performed at 1 °C.

X-ray Diffraction. Single crystals of the 569/H/9 Zn-β-lactamase were obtained at room temperature by vapor diffusion in hanging drops at pH 5.6 as described previously (*1*). Those crystals were rapidly transferred to a HEPES buffer solution, pH 7.5. X-ray diffraction data were collected at 100 K to 2.0 Å resolution after flash cooling of the crystals in HEPES buffer, using a MARResearch image-plate detector and Cu Kα radiation from a Rigaku rotating-anode generator (40 kV, 100 mA). The crystals belong to space group *C*2 with $a = 52.84$ Å, $b = 60.95$ Å, $c = 138.53$ Å, $\beta = 93.11°$ and 2 molecules in the asymmetric unit.

PAC Spectroscopy. The PAC spectrometer is described in ref *13* and references therein. In the case of identical, static and randomly oriented molecules, the perturbation function denoted $G_2(t)$ can be written

$$G_2(t) = a_0 + a_1 \cos(\omega_1 t) + a_2 \cos(\omega_0 t) + a_3 \cos(\omega_3 t)$$

where ω_1, ω_2, and ω_3 are the three difference frequencies between the three sublevels of the spin $^5/_2$ state (*14*). Note

Table 1: Number of Cadmium Ions Bound per Enzyme Molecule for the 5/B/6 and 569/H/9 Wild Type and the Cys168Ala Mutant Enzymes[a]

	[Cd] (μM)	Cd ions bound/enzyme molecule
5/B/6	10	1.6
	50	1.8
569/H/9 WT	10	1.0
	48	1.2
569/H/9 C168A mutant	5	1.1
	47	1.2

[a] As measured by AAS after dialysis of the apoenzymes (below 1 μM) against the given [Cd] in 25 mM HEPES buffer, pH 7.5, and 1 M NaCl. The zinc content was always below 12% as checked by AAS. The standard deviations are 0.1 Cd(II) per enzyme molecule.

that $\omega_1 + \omega_2 = \omega_3$. Thus the Fourier transform of $G_2(t)$ exhibits three frequencies for each NQI.

The NQI is described by the numerically largest diagonal element after diagonalization, chosen as $|\omega_{zz}|$, which is denoted ω_0, and $\eta = |(\nu_{xx} - \nu_{yy})/\nu_{zz}|$. The relation between these two parameters and the frequencies in eq 2 can be found in Bauer (*14*). Thus from the time dependence of $G_2(t)$, ω and η can be determined through least-squares fitting.

In the liquid state the nuclear quadrupole interaction is time-dependent because of the Brownian reorientation of the protein, described by the rotational diffusion time τ_R. This has the consequence that $G_2(t)$ converges to 0 as a function of time, representing thermal equilibrium and isotropy in the angular correlation between the two γ-rays. The effect of rotational diffusion is described in Danielsen et al. (*15*).

PAC Data Analysis. The perturbation function $A_2G_2(t)$, was analyzed by a conventional nonlinear least-squares fitting routine. Satisfactory fitting was obtained with a relative Gaussian distribution $\delta = \Delta\omega/\omega$ applied to all the three frequencies. Finite values for δ indicate that the ^{111m}Cd nuclei are located in a distribution of surroundings. A NQI is then described by the parameters ω_0, η, δ, and τ_R. In cases where more than a single NQI is present, the perturbation function is the sum of the different perturbation functions, where each NQI is weighted by its population (*13*).

RESULTS

Binding of Cd(II) to Different Bacillus cereus Enzyme Species. Table 1 shows the number of cadmium ions bound per enzyme molecule, found after equilibrium dialysis of different apoenzyme species against different Cd concentrations. For the apoenzyme of strain 569/H/9 and the corresponding C168A mutant only slightly more than one cadmium ion was bound even at the highest cadmium ion concentration. Thus it appears that only one of the two metal sites binds cadmium ions with high affinity (dissociation constant less than 10 μM). This is in contrast to results obtained with Zn(II) for all three enzymes (*10*) and with Cd(II) for the 5/B/6 enzyme, where both metal ions bind with dissociation constants below or close to 10 μM.

The results of dialysis experiments performed at low stoichiometry between Cd(II) and the 5/B/6 apo-β-lactamase gave dissociation constants at pH 7.5, K_{mono}, for Cd(II) binding to BCII (5/B/6) of 0.3 ± 0.1 μM and 0.5 ± 0.3 μM with and without 1 M NaCl, respectively [derived from four different enzyme concentrations: 50 mM HEPES, pH 7.5 (0.36, 0.66, 1.34, and 2.69 μM apoenzyme) and 50 mM HEPES, pH 7.5, and 1 M NaCl (0.27, 0.55, 1.09 and 2.18 μM apoenzyme) with the concentration of ^{109}Cd being 1.2 nM in the total volume].

Table 2: Kinetic Parameters of Cd(II) Derivatives of the 5/B/6 Enzyme[a]

	mononuclear Cd enzyme[b]			binuclear Cd enzyme		
[NaCl] (mM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} s^{-1})
no salt	8.8	24	0.37	130	38	3.4
25	7.4	45	0.16	68	60	1.1
50	8.0	49	0.16	40	53	0.75
100	9.3	67	0.14	21	76	0.28
250	8.3	100	0.08	13	60	0.22
1000	7.4	91	0.08	15	53	0.28

[a] In 25 mM HEPES, pH 7.5, with nitrocefin as substrate and in the presence of increasing concentrations of NaCl. The enzyme samples were prepared as described in the text. The kinetic parameters were derived from analysis of initial rates of hydrolysis. SD values are below 10%. [b] See text for a description of what we interpret as being the mononuclear Cd(II) enzyme.

Binding experiments with an excess of cadmium ions relative to the 5/B/6 apoenzyme showed that at least 2 moles of Cd(II) can bind per mole of apoenzyme in 25 mM HEPES buffer, pH 7.5. The dissociation constant K_{bi} was 1 μM; the standard deviations were different for an increase relative to a decrease in K_{bi} and equal to 2 and 0.6 μM, respectively, with 1 M NaCl (10.5, 21.0, 42.0, and 84.0 μM Cd(II) dialyzed against 11.6 μM enzyme in 50 mM HEPES, pH 7.5, and 1 M NaCl).

In the absence of NaCl, the quality of the data was not sufficiently good to allow the determination of K_{bi}.

Dialysis of the native 5/B/6 Zn(II) enzyme against buffers containing 10−100 μM Cd(II) always resulted in 1.0 ± 0.1 bound cadmium ion as well as 1.0 ± 0.1 bound zinc ion per enzyme molecule, suggesting that in the mixed Zn,Cd species the zinc and cadmium ions selectively occupy different sites. Similar results were obtained with the *B. fragilis* metallo-β-lactamase (*16*).

Hydrolysis of Nitrocefin by Cd-Substituted 5/B/6 β-Lactamase. In the presence of 100 μM Cd(II) the enzyme binds 1.7−2.0 equiv of Cd(II) as shown by AAS. Therefore kinetic parameters obtained under these conditions likely represents the Cd_2E species. Dilution (100-fold) of the binuclear Cd_2E in 150 μM nitrocefin, and 10 μM EDTA resulted in significantly reduced activity, which can be attributed to a loss of bound Cd(II). The remaining activity was unchanged during a period of 5−10 min, suggesting that EDTA extracted one of the two bound cadmium ions, making it likely that the enzyme appears preferentially as the mononuclear CdE species. Similar results have been obtained with the Zn(II)-containing forms of the enzyme (*10*). Table 2 presents the kinetic parameters characteristic of the EDTA-treated assumed mononuclear and likely saturated binuclear Cd(II) enzymes at different NaCl concentrations. The k_{cat} value of the assumed mononuclear Cd(II) enzyme shows no dependency on NaCl concentration but the K_m value increases by a factor of 4 upon addition of 1 M NaCl. However, a dramatic effect of NaCl for the binuclear enzyme is seen by the k_{cat} value decreasing by a factor of 10 when the

Table 3: Kinetic Parameters of Mono- and Binuclear 5/B/6 Zn(II) Enzymes, Mononuclear Cd Derivative, Binuclear Cd Derivative, and Mixed Zn,Cd Species[a]

	no NaCl			[NaCl] > 250 mM		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} s^{-1})
Zn- and Zn_2E	29	43	0.67	60[b]	78[b]	0.77
Zn,CdE	61	28	2.2	99[b]	150[b]	0.65
CdE[c]	8.8[d]	24[d]	0.37	7.9[e]	96[e]	0.08
Cd_2E	130	38	3.4	14[e]	56[e]	0.25

[a] In in 25 mM HEPES, pH 7.5, with nitrocefin as substrate in the presence or absence of NaCl. SD values are below 10%. [b] Determined in the presence of 1 M NaCl. [c] See text for a description of what we interpret as being the mononuclear Cd(II) enzyme. [d] From Table 2. [e] Average of the values in the presence of 0.25 and 1 M NaCl from Table 2.

Table 4: NQI Parameters for ^{111m}Cd-β-Lactamase from Strain 5/B/6

NQI	ω_0 (Mrad/s)	η	δ	τ_R (ns)
NQI-c1[a]	172 ± 2	0.34 ± 0.02	0.14 ± 0.01	128 ± 4
NQI-c2[a]	95 ± 5	0.47 ± 0.10	0.14 ± 0.01	128 ± 4
NQI-e1[b]	141 ± 3	0.87 ± 0.08	0.05 ± 0.01	28 ± 3
NQI-e2[b]	76 ± 2	0.54 ± 0.09	0.05 ± 0.01	28 ± 3

[a] Carrier-free conditions, fit to the added spectrum for pH 6.3, 7.4, 7.9, and 8.0 (see Table 5). [b] Carrier conditions, fit to the spectrum with 1.5 equiv of Cd and NaCl present (see Table 8).

concentration of NaCl is increased without any substantial change in K_m.

The kinetic parameters for the hybrid Zn(II), Cd(II) species (Table 3) were obtained with samples where 1 ± 0.1 cadmium ion as well as zinc ion was bound per enzyme molecule as determined by AAS. The K_m and k_{cat} values were the same for Zn,CdE when the assays were performed in metal-free buffer or in the presence of 5 μM EDTA or 50 μM Cd(II), indicating that the dilution of the enzyme for kinetic measurements did not change the metal content of the enzyme in contrast to the measurements with Cd_2E. For the native Zn(II) enzyme, the hydrolysis of nitrocefin (Table 3) is identical for the mononuclear and binuclear Zn(II) enzyme (*10*).

PAC Spectra of the Cd(II) Enzyme from Strain 5/B/6 (Carrier-Free Conditions). The added PAC spectra obtained with excess of apoenzyme (7−50 μM) compared to the concentration of Cd(II) (less than 0.1 μM) was used to derive the NQIs (NQI-c1 and NQI-c2 in Table 4). The added spectrum at low stoichiometry of Cd(II):enzyme is shown in Figure 1. All individual PAC spectra under carrier-free conditions were fitted with fixed NQIs as derived from the added spectrum for strain 5/B/6. The abundance of these NQIs as well as the abundance of non-protein-bound Cd(II) is shown in Table 5. A PAC experiment performed at low stoichiometries of Cd(II):enzyme carried out at 30 °C showed the same relative abundance between NQI-c1 and NQI-c2 as shown in Table 5 for 1 °C. Sucrose is not expected to have any effect on the relative abundance of the different NQIs as the known effect of sucrose most often is to stabilize the global structure of proteins without interfering with the active-site structure. Only the spectrum taken at pH 5.6 shows a significant fraction of non-protein-bound Cd(II). From this, K_{mono} was calculated as 5.6 ± 0.7 μM at pH 5.6, using 7 μM for the BCII concentration. At pH 6.3 and higher, K_{mono} is less than 1 μM, in agreement with the result from dialysis. From Table 5 it is seen that two NQIs are simultaneously present, the dominating NQI being NQI-c1. The ratio between the amount of NQI-c1 to the amount of NQI-c2, having a value of 3 on average, is affected neither by pH nor by NaCl concentration, except at the highest pH without NaCl present, where NQI-c1 dominates completely (Table 5).

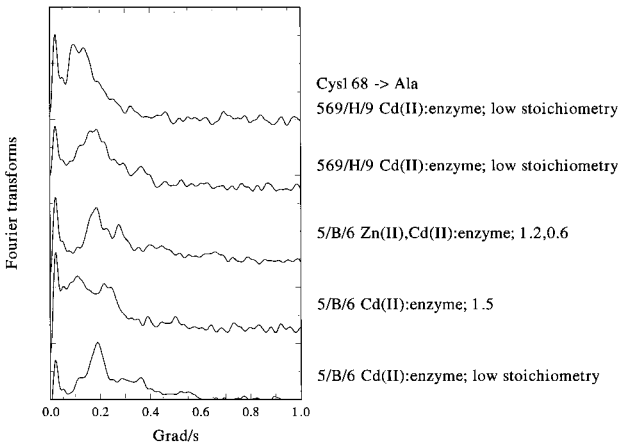

FIGURE 1: Fourier transform of various PAC spectra.

Table 5: Populations of NQI Parameters for ^{111m}Cd-β-Lactamase for Strain 5/B/6 under Carrier-Free Conditions

pH	NaCl (mM)	BCII (μM)	NQI-c1 (%)	NQI-c2 (%)	Cd (% npb[a])	χ_r^2
5.6	10	7	39 ± 3	16 ± 3	44 ± 3	1.12
6.3	11	8	78 ± 3	26 ± 3	−4 ± 3	1.03
7.4	35	8	76 ± 3	26 ± 3	−2 ± 3	1.25
7.9	0[b]	65	75 ± 3	21 ± 3	4 ± 3	1.14
8.0	370	29	80 ± 3	20 ± 3	0 ± 3	1.16
9.2	0[b]	26	95 ± 3	6 ± 3	−1 ± 3	1.11

[a] npb, non-protein-bound. [b] [NaCl] ≤ 2 mM.

PAC Spectra Obtained from the Cd(II) and the Zn(II), Cd(II) Enzyme from Strain 5/B/6 (Carrier Conditions). In these measurements radioactive ^{111m}Cd was mixed with nonradioactive Cd(II) before addition to the apoenzyme. For measurements on the Zn(II), Cd(II) enzyme, Zn(II) was added before Cd(II).

(A) With NaCl Present. The spectrum containing Cd(II) in a ratio of 1.5 between Cd(II) and apoenzyme was used to determine the NQIs present under carrier conditions (Figure 1). The NQIs, denoted NQI-e1 and NQI-e2, derived from least-squares fits are presented in Table 4. The PAC spectra obtained under carrier-free conditions (Figure 1) are different from those obtained under carrier conditions (Figure 1). Correspondingly, the two NQIs (NQI-c1 and NQI-c2) are different from the NQIs denoted NQI-e1 and NQI-e2 (Table 4). The PAC spectrum obtained in the presence of Zn(II) at a ratio of 1.2 between Zn(II) and apoenzyme and simultaneously in the presence of Cd(II) at a ratio of 0.6 between Cd(II) and apoenzyme is very different from the spectrum with 1.5 mol of Cd(II) relative to the apoenzyme (Figure 1). The corresponding χ^2 fit showed that the spectrum for the mixed Zn(II), Cd(II) enzyme could, besides non-protein bound Cd(II), be analyzed with only one NQI (NQI-e3) having a relatively high value for ω_0 (Table 6). In Table 7 the populations of the different NQIs for various ratios of Cd(II):apoenzyme are shown. For the four ratios given in

Table 6: NQI Parameters for ^{111m}Cd-β-Lactamase from Strain 5/B/6 in the Presence of Zn(II)[a]

NQI	ω_0 (Mrad/s)	η	δ	τ_R (ns)	Cd (% npb[b])
NQI-e3	142 ± 1	0.51 ± 0.01	0.13 ± 0.01	114 ± 40	14 ± 3

[a] Experiments performed with 50 μM apo-β-lactamase in 370 mM NaCl and with 1.2 mol of Zn(II) and 0.6 mol of Cd per apo-β-lactamase. [b] npb, non-protein-bound.

Table 7 (in the presence of NaCl), the ratio between the population of NQI-e1 and NQI-e2 is always close to 1. The protein-bound Cd(II) presented in the last column shows clearly that 2 and only 2 mol of Cd/mol of enzyme can be bound at the protein concentrations used. For the three lowest [Cd]/[E] ratios the populations of NQI-e1 and NQI-e2 increase with increasing [Cd]/[E] ratio as expected. For the [Cd]/[E] ratio of 3.0 this does not seem to be the case. However, the large fraction of non-protein-bound Cd(II) in this case partly obscures the interpretation of the spectrum.

(B) Without NaCl Present. Two spectra were obtained without NaCl but with stoichiometric amounts of Cd(II) [1.5 and 1.9 mol of Cd(II)/mol of enzyme]. In the presence of 1.5 and 1.9 of mol Cd(II)/mol of enzyme the amount of protein-bound Cd(II) is about 1.0 and 1.4 Cd(II)/enzyme, respectively (Table 7), clearly indicating a weaker binding of a second equivalent of Cd(II) in the absence of NaCl (K_{bi} larger than 50 μM). By analyzing the PAC spectra with the same set of NQIs found in the presence of NaCl, the populations of NQI-e1 and NQI-e2 were both close to zero (Table 7), suggesting that these signals occur only in the presence of NaCl. No attempt was made to include other NQIs as representing the binuclear species, due to the relatively poor quality of the spectra.

PAC Spectra of the Cd Enzyme from Strain 569/H/9 Including the Cys168 → Ala Mutant. A spectrum from the 569/H/9 enzyme at pH 7.7 at low stoichiometry of Cd(II) relative to the apoenzyme (Figure 1) gave similar NQI parameters as the spectra from 5/B/6 (Tables 4 and 8). The difference may be explained by small changes in ligand positions (within 0.1 Å). For the Cys168 → Ala mutant from strain 569/H/9 the high-frequency NQI (NQI-c3) is absent (Table 8 and Figure 1), identifying this NQI as arising from a metal coordination geometry having Cys168 as a ligand. With this assignment it follows that the Cd(II) site having Cys168 as a ligand has the lowest dissociation constants (NQI-c1 in Table 5). In the presence of Cd(II) at a ratio of 1.9 relative to the apoenzyme, 1.0 mol of Cd(II) binds/mol of apoenzyme for the Cys168 → Ala mutant of strain 569/H/9 (Table 8), indicating that the three-His site can still be fully occupied. In the presence of Cd(II) at a ratio of 1.9 relative to the WT apoenzyme, only about 1.4 mol of Cd(II) is bound/mol of apoenzyme. When such a large fraction of the cadmium ions are non-protein-bound, it is not possible to identify two new NQIs characteristic of the binuclear enzyme. Therefore, the populations of the two NQIs c3 and c4 are probably incorrect. However, the number of cadmium ions bound per mole of apoenzyme is always reliably deduced. The dissociation constant for the binuclear enzyme can therefore be calculated as 40 ± 10 μM, based on the value for the monosubstituted enzyme being less than 1 μM.

This shows that the dissociation constant of cadmium ions from the binuclear form of the 569/H/9 strain is higher than for the 5/B/6 strain in the presence of NaCl.

Metal Sites Coordination Geometry from X-ray Diffraction Data. In the present work we present the structure of the 569/H/9 enzyme at the metal ion binding sites. The full

Table 7: Populations of NQI Parameters for ^{111m}Cd-β-Lactamase from 5/B/6 for Different Cd:BCII Ratios

added Cd	NQI-c1	NQI-c2	NQI-e1	NQI-e2	protein-bound Cd
			With NaCl[a]		
0.8	0.26 ± 0.03	0.09 ± 0.03	0.21 ± 0.03	0.19 ± 0.03	0.75 ± 0.03
1.5	0.20 ± 0.06	0.18 ± 0.06	0.47 ± 0.05	0.59 ± 0.06	1.43 ± 0.05
2.2	0.31 ± 0.13	0.13 ± 0.13	0.62 ± 0.09	0.70 ± 0.11	1.74 ± 0.07
3.0	0.39 ± 0.15	0.48 ± 0.15	0.45 ± 0.09	0.57 ± 0.12	1.89 ± 0.12
			Without NaCl[a]		
1.5	0.34 ± 0.06	0.44 ± 0.05	0.14 ± 0.06	0.11 ± 0.06	1.03 ± 0.06
1.9	0.48 ± 0.06	0.74 ± 0.06	0.12 ± 0.06	0.03 ± 0.06	1.38 ± 0.05

[a] The NaCl concentration was between 250 and 370 mM with NaCl and below 2 mM without. The apoenzyme concentration was between 33 and 65 μM. The pH was 8 within 0.2 unit. All columns are in units of moles of Cd per mole of enzyme.

Table 8: NQI Parameters and Populations for ^{111m}Cd-β-Lactamase from Strain 569/H/9

		NQI Parameters, Carrier-Free			
enzyme	NQI	ω_0 (Mrad/s)	η	δ	τ_R (ns)
WT	NQI-c3	149 ± 9	0.42 ± 0.04	0.22 ± 0.04	72 ± 12
WT	NQI-c4	84 ± 11	0.50 ± 0.14		

	Population of NQIs, Carrier-Free[a]		
enzyme	NQI-c4 (%)	NQI-c3 (%)	Cd (% npb[b])
WT	30 ± 3	70 ± 3	0 ± 3
Cys168 → Ala mutant	89 ± 3	1 ± 3	11 ± 3

	Population of NQIs, with a Ratio of 1.9 for [Cd]/[E][a]		
enzyme	NQI-c4	NQI-c3	protein-bound Cd
WT	0.76 ± 0.06	0.55 ± 0.06	1.37 ± 0.06
Cys168→Ala mutant	0.87 ± 0.06	0.11 ± 0.08	0.99 ± 0.06

[a] The NaCl concentration was between 270 and 350 mM, the apoenzyme concentration was 50 μM, and the pH was 7.6 within 0.2 unit. [b] npb, non-protein-bound.

Table 9: Angles and Distances in the Metal Cluster of the *B. cereus* II Zn-β-Lactamase Molecule with the Bridging Water[a]

Ligand−Zinc−Ligand Angles (in Degrees)			
three-His site		Cys site	
ligand pair	angle	ligand pair	angle
His 86, His 88	104.0	Asp 90, Cys 168	99.6
His 86, His 149	104.4	Asp 90, His 210	82.3
His 86, H_2O	112.0	Asp 90, H_2O	98.0
His 88, His 149	119.3	Cys 168, His 210	110.8
His 88, H_2O	105.1	His 210, H_2O	136.0

Ligand Distances to Zinc (in Angstroms)							
three-His site				Cys site			
His86	His88	His149	H_2O	Asp90	Cys168	His210	H_2O
2.04	2.06	2.07	2.19	2.15	2.15	2.25	2.19

[a] From the X-ray structure at pH 7.5.

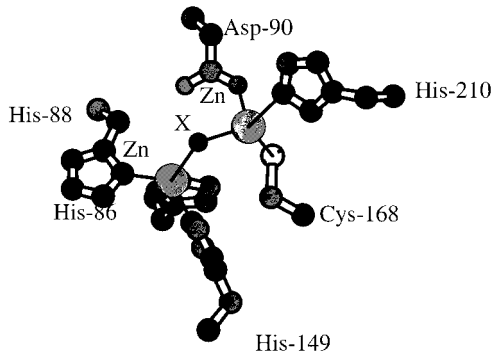

FIGURE 2: Metal coordination geometries of the *B. cereus* II Zn-β-lactamase molecule with the bridging solvent molecule denoted X (from the X-ray structure at pH 7.5 presented in this work).

structure will be published later. The refined structure reveals a binuclear metal center. Surprisingly, in one molecule of the asymmetric unit a water molecule is bridging the two zinc ions, while it is absent in the second molecule. For the molecule with the bridging water, the coordination geometry of the metal sites is described in Table 9 and Figure 2. The distance between the two zinc ions is 3.94 Å.

DISCUSSION

In the present work, we established the existence of two unique Cd(II) coordination geometries belonging to different metal ion binding sites. Furthermore, it was possible via the PAC spectrum from the Cys168 → Ala mutant to assign one NQI to the Cys site (high ω_0 values, NQI-c1, NQI-e1, and NQI-e3) and the other to the three-His site (low ω_0 values, NQI-c2 and NQI-e2). These assignments infer that the site involving Cys168 has the strongest affinity for Cd(II) (Table 5). This is in contrast to zinc ions in the Zn(II)), Cd(II) hybrid enzyme, which blocks the three-His sites for Cd(II) (Table 6). Therefore, according to our assignment of NQIs to metal coordination geometries, a cysteine residue is not involved as a ligand for the three-His site in the Cd(II) enzyme, as has been suggested for both zinc and cadmium (*4*), but a charge transfer band in UV spectroscopy from a metal-coordinated cysteine residue appears simply because cadmium ions preferentially occupies the Cys site also at Cd(II): enzyme stoichiometries below 1. In conclusion, for Cd(II): enzyme stoichiometries below 1 there is an equilibrium between the two metal sites for cadmium ions favoring the Cys site by 3:1 (Table 5). For the zinc enzyme where the three-His site is likely preferred, at least in the presence of Cd(II) ions, a similar equilibrium likely exists with the ratio for the populations of the Cys site to the three-His site being less than 1. In agreement with this, a partial occupation of the Cys site at stoichiometries of Zn(II) to the enzyme close to 1 was derived from EXAFS spectroscopy (*10*).

That the two NQIs NQI-e1 and NQI-e2 are different from NQI-c1 and NQI-c2 leads to the immediate conclusion that the two metal sites interact with one another. Furthermore, the unexpectedly high population of NQI-e1 and NQI-e2 at a [Cd]/[E] ratio of 0.8 (Table 7) suggests positive cooperativity in the binding of two cadmium ions with NaCl present. The obvious way to interact, in the absence of a common acidic residue ligand, is to have a bridging solvent molecule between the two cadmium ions, as was found for Zn(II) in the enzyme from *B. cereus* (X-ray data, this work) and in the enzyme from *B. fragilis* (*7*). From a chemical point of view a negatively charged ion is probably the most favorable choice as the solvent molecule because of the two formally doubly positively charged metal ions. Furthermore, as we only observe metal−metal interaction in the presence of NaCl, a Cl^- ion is the obvious choice. The fact that we cannot demonstrate interaction between the two metal sites in the absence of NaCl or for the 569/H/9 strain of the enzyme does not exclude a bridging ligand in these cases, but the effect on the NQIs appears to be below the detection limit. Note, however, that in these two cases PAC data exist with at least a partial population of the binuclear enzyme (Table 7). In this connection it is important to note that the NQIs c2 and e2 have values close to the values for the low-pH form for Cd(II) in carbonic anhydrase but are quite far away from the values for the corresponding high-pH form, which was interpreted as a ligand geometry consisting of three histidine and one OH^- ligands (*17*). Furthermore, calculations of the NQI for the three-His site, using the semiempirical angular overlap model (*18*) and the structure determined by X-ray diffraction (this work), cannot reproduce the measured NQI for the three-His site with an OH^- as solvent ligand. Thus if an OH^- ligand is involved when no NaCl is present at the three-His site it must, for example, be in a five-coordinated geometry with one water and one OH^- ligand.

In the β-lactamases from *B. cereus* as well as from *B. fragilis* there are two zinc sites within 4 Å of each other (from X-ray data in this work and refs *5*−*7*). However, in

the work of Carfi et al. (*1*) only one zinc ion was found. Furthermore, it was shown that for *B. cereus* only one zinc ion bound to the enzyme is needed for full activity of nitrocefin hydrolysis (*10*). Thus, despite the exciting discovery of two bridged zinc ions in β-lactamase, the chemical consequences as well as any possible biological benefit of two connected zinc ions is unclear. For the enzymes where only one zinc ion is needed for full activity (*10*, *16*), the question arises, in which of the two above-mentioned metal coordination geometries Zn(II) is situated. Conserved residues and X-ray studies demonstrate the prominence of a three-His zinc site. The exclusive presence of Cd(II) in the Cys site when 1 equiv of Zn(II) is present further shows a preference for Zn(II) at the three-His site. With few exceptions the three residues involved in the other zinc site, namely, Asp, His, and Cys, are also conserved in metallo-β-lactamases. Assuming that the kinetic results (Table 3) from the EDTA-treated enzyme represents an enzyme with 1 or less mol of Cd(II)/mol of enzyme, which we denote the mononuclear Cd(II) enzyme, the PAC results combined with the low value for k_{cat} for the mononuclear Cd(II) enzyme relative to the values for the binuclear enzyme suggest that the three-His site is the primary catalytic site and the Cys site is probably a cocatalytic site (*19*).

The marked effect of NaCl on k_{cat} for the binuclear enzyme absent for the assumed mononuclear enzyme suggests that for the binuclear enzyme an additional metal ion ligand (likely Cl^-) site exists, reducing but not abolishing catalytic activity. A three-dimensional structure from X-ray diffraction on the Cd_2E species at NaCl concentration above 100 mM could reveal whether this is the case. The kinetic parameters for the binuclear Cd(II) enzyme at high salt concentration is close to that of the assumed mononuclear Cd(II) enzyme. As suggested by many groups (ref *20* and references therein), the solvent ligand in the three-His site is the group responsible for the attack on the β-lactam ring. The enhanced activity for the binuclear Cd(II) enzyme at low concentration of NaCl could be an effect of the lowering of a p*K* for a cadmium-bound water molecule, when the water molecule bridges two cadmium ions.

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