Dynamics of Mononuclear Cadmium β-Lactamase Revealed by the Combination of NMR and PAC Spectroscopy

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Abstract: The two metal sites in cadmium substituted β-lactamase from *Bacillus cereus* 569/H/9 have been studied by NMR spectroscopy (^{1}H, ^{15}N, and ^{113}Cd) and PAC spectroscopy (^{111m}Cd). Distinct NMR signals from the backbone amides are identified for the apoenzyme and the mononuclear and binuclear cadmium enzymes. For the binuclear cadmium enzyme, two ^{113}Cd NMR signals (142 and 262 ppm) and two ^{111m}Cd PAC nuclear quadrupole interactions are observed. Two nuclear quadrupole interactions are also observed, with approximately equal occupancy, in the PAC spectra at cadmium/enzyme ratios < 1; these are different from those derived for the binuclear cadmium enzyme, demonstrating interaction between the two metal ion binding sites. In contrast to the observation from PAC spectroscopy, only one ^{113}Cd NMR signal (176 ppm) is observed at cadmium/enzyme ratios < 1. The titration of the metal site imidazole (N)H proton signals as a function of cadmium ion-to-enzyme ratio shows that signals characteristic for the binuclear cadmium enzyme appear when the cadmium ion-to-enzyme ratio is between 1 and 2, whereas no signals are observed at stoichiometries less than 1. The simplest explanation consistent with all data is that, at cadmium/enzyme ratios < 1, the single Cd(II) is undergoing exchange between the two metal sites on the enzyme. This exchange must be fast on the ^{113}Cd NMR time scale and slow on the ^{111m}Cd PAC time scale and must thus occur in a time regime between 0.1 and 10 μs.

Introduction

β-Lactamases make bacteria resistant to β-lactam antibiotics by cleaving the β-lactam C−N bond and inactivating these drugs.[1] The zinc containing metallo-β-lactamases are becoming an increasingly worrying clinical problem, because no clinically useful inhibitors of these enzymes are available. The development of inhibitors requires an understanding of the enzymes on the atomic level and, particularly, of the structure and dynamics of their essential metal ion binding sites.

A number of structures of these enzymes, including both mono- and binuclear zinc enzymes, have been determined by X-ray diffraction, revealing two potential zinc ion binding sites at the enzyme's active site.[2−11] The precise function of the two metal ions during catalysis remains unsettled, although a number of mechanistic suggestions have been put forward.[1,12−14] In the resting state of the enzyme, the most likely metal ligands seem to be three histidines (86, 88, and 149 (or 116, 118, and 196 in the class B β-lactamase (BBL) numbering,[15] which will be given in brackets henceforth)) and a solvent molecule at one site (site 1) and a histidine (210 (BBL 263)), a monodentate aspartic acid, a cysteine, and one or two solvent molecules at the other (site 2). The two metal ions are found relatively close to each other, at a distance of 3.7−4.4 Å for the enzyme from *B. cereus*,[3−5] 3.4−3.8 Å for the enzyme from *B. fragilis*[6−9] (in the 2.5 Å resolution structure of the *B. fragilis* enzyme,[10] the zinc−zinc

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distances are 3.2 and 2.8 Å for the two independent molecules), and 3.5−3.6 Å for the IMP-1 enzyme.[11] Several structures of the *B. fragilis* enzyme show a bridging ligand[6,7,9] between the two metals, suggested to be OH^-; however, this bridging solvent molecule is not present in other structures of the *B. fragilis* enzyme[8,10] and is closely associated only to the zinc in site 1 in the binuclear structures of the *B. cereus* enzyme.[3,4] In one structure, the bridging solvent molecule is present in only one of the two molecules in the asymmetric unit.[5] The second solvent molecule at site 2 is carbonate in one structure,[3] water at very variable zinc−oxygen bond lengths (2.18−2.69 Å) in other structures,[4,6−9,] or even missing.[5,10,11] Thus, the structures show great variability in the coordination of the two metal ions.

The affinity of the enzyme for the metal ions differs between β-lactamases from different bacteria.[16−18] The first metal ion is bound with high affinity by the enzymes from both *B. fragilis* and *B. cereus*, but the second metal ion is bound more weakly in the latter.[16] The enzyme is active with either one or two zinc ions bound, however, with different kinetic characteristics.[5,19] Zinc can be exchanged with cadmium, and both the mono- and binuclear cadmium enzymes are catalytically active.[5] Interestingly, recent ^{111m}Cd PAC spectroscopic results performed in solution on β-lactamase from *B. cereus* indicated that, even at low [Cd(II)]/[E] ratios, either of the two sites could be occupied.[5] That is, some enzyme molecules had one site occupied and others had the other site occupied, but all were mononuclear cadmium enzymes.

To investigate further the coordination properties of the two metal sites in β-lactamases, we have performed ^{1}H, ^{15}N, and ^{113}Cd NMR and ^{111m}Cd PAC spectroscopy on cadmium substituted β-lactamase from *B. cereus*. ^{113}Cd NMR has previously been applied to several different cadmium substituted zinc enzymes, and the chemical shift has been shown to be sensitive to the nature and number of the metal ion ligands.[20] We have previously reported the identification of the imidazole ^{1}H and ^{15}N resonances from the histidine metal ligands.[21] Together, these NMR methods provide a valuable approach to characterizing cadmium binding to the enzyme. PAC spectroscopy has been successfully applied to the study of a number of metal ion containing proteins.[5,22−25] It provides information on the metal ion coordination geometry, through measurement of the nuclear quadrupole interaction (NQI) between the nuclear electric quadrupole moment of the metal and the electric field gradient from the surrounding charge distribution.

In this work, we combine the two experimental techniques PAC and NMR in order to shed light on the binding of one or two metal ions to the metallo-β-lactamase from *B. cereus* and show that the combined application of the two methods provides unique information on the dynamical features of the metal ion binding sites.

Materials and Methods

Enzyme Preparation. ^{15}N-Labeled protein was expressed from plasmid pET/BCII in *E. coli* BL21(DE3). Cells were grown at 30 °C in M9 minimal medium with 4 g of glucose and 1 g of $^{15}NH_4Cl$ as the only nitrogen source. Expression was induced by adding 0.5 mM IPTG (isopropyl-β-thiogalactoside) at an absorbance at 600 nm of 1.25. After 16 h, the cells were harvested by centrifugation and were broken by sonication, and the β-lactamase was purified as described elsewhere.[2] The apoenzyme was prepared as described by Paul-Soto et al.[19] For the NMR studies, the cadmium enzyme was prepared at room temperature, by adding gradually a small volume (1−10 μL) of 0.1 M $^{113}CdCl_2$ (95.83% enriched) to the β-lactamase apoenzyme (0.8−3.0 mM) in 20 mM MES-Na, 100 mM NaCl, pH 6.4, 5% D_2O. The final sample volume was 450 mL, and the temperature 298 K. The same enzyme preparation and similar sample preparation were used in PAC spectroscopy (see below).

Determination of Dissociation Constants for Cadmium Binding. All experiments were performed in 15 mM HEPES pH 7.0 at 25 °C. The macroscopic dissociation constants for the binding of the first and second cadmium ions (K_d', K_d'') were obtained from competitive titrations with the chromophoric chelator Mag-fura-2 (Molecular Probes, Eugene, Oregon). The indicator was titrated with cadmium in the absence and presence of the apoenzyme (protein/indicator ratios were between 1:1 and 1:3). Changes in absorbance at 363 nm were monitored.[26] The program Chemsim (developed by R. Kramer, Saarbrücken) was used for numerical data analysis. The program allows analysis of binding experiments involving coupled equilibria.[27] For calculations involving coupled equilibria, a combination of a Simplex algorithm with a Newton−Raphson procedure was used to minimize the sum of least squares. The dissociation constants obtained for the Mag-fura-2−Cd(II) complex were 47 nM (without salt) and 127 nM (with 0.1 M NaCl).

NMR Spectroscopy. All NMR experiments were performed on Bruker Avance DRX/DMX instruments. One-dimensional ^{1}H spectra were obtained at 600 MHz by using a water-flip-back pulse combined with Watergate.[28,29] Backbone NH resonances were observed by [^{1}H,^{15}N]-HSQC (heteronuclear single quantum coherence) with gradient coherence selection and sensitivity enhancement.[30]

All ^{113}Cd experiments were performed at 133 MHz, with a 5 mm reverse broad-band probe. About 100 000 free induction decays were recorded, acquiring 4096 complex points in 51 ms, with a 30° observe pulse (4 μs) and a spectral width of 80 000 Hz (600 ppm), and with a relaxation delay of 0.5 s. A 300 Hz exponential multiplication was used prior to Fourier transformation, followed by baseline correction. The external reference was $Cd[ClO_4]_2$.

PAC Spectroscopy. The ^{111m}Cd was produced by the Cyclotron Department at the University Hospital in Copenhagen. Preparation and purification of ^{111m}Cd was described previously.[23] Solution volumes of 10 or 40 μL containing about 10 or 40 pmol ^{111m}Cd in H_2O were mixed with 40−100 μL of metal-depleted MES buffer containing various stoichiometric amounts of cadmium ions. (Other contaminating metal ions were present at concentrations of 0.1 μM or less in the final sample, at least 100-fold lower than the cadmium ion and enzyme concentrations used in the experiments.) To these solutions was added apoenzyme such that the final enzyme concentration was 0.5 mM. The pH was adjusted at room temperature to pH 6.0 unless otherwise stated, by addition of small amounts of 1−5 M HCl or NaOH. The final sample

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conditions were 20 mM HEPES and 0.1 M NaCl, and volumes ranged from 50 to 200 μL. A 10 min waiting period (at room temperature) allowing the metal(s) to bind was included before sucrose was added to give a 50% w/w solution and the sample was cooled to 1 °C. All PAC experiments were performed at 1 °C.

The PAC spectrometer is described in Bauer et al.[24] and references therein. The perturbation function, $G_2(t)$, is derived from the measurements as described previously.[22−25] This function contains the structural information about the coordination geometry that can be deduced from a PAC experiment. In the case of identical, static, and randomly oriented molecules, the perturbation function denoted $G_2(t)$ can be written

$$G_2(t) = a_0 + a_1 \cos(\omega_1 t) + a_2 \cos(\omega_2 t) + a_3 \cos(\omega_3 t)$$

where ω_1, ω_2, and ω_3 are the three difference frequencies between the three sublevels of the spin 5/2 state of the cadmium nucleus.[22] Note that $\omega_1 + \omega_2 = \omega_3$. Thus, the Fourier transform of $G_2(t)$ exhibits three frequencies for each NQI. In this work, the Fourier transformation was performed as described in Bauer et al.[24] The NQI is described by the numerically largest diagonal element after diagonalization of the NQI tensor, chosen as ω_{zz}, which is denoted ω_0, and $\eta = (\omega_{xx} - \omega_{yy})/\omega_{zz}$ (with $|\omega_{xx}| \leq |\omega_{yy}| \leq |\omega_{zz}|$). The relation between these two parameters and the frequencies in $G_2(t)$ can be found in Bauer.[22] Thus, ω_0 and η, which reflect the coordination geometry of the cadmium ion, can be determined from the time dependence of $G_2(t)$ through least-squares fitting.

In the liquid state, the NQI 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 zero 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.[31]

PAC Data Analysis. The perturbation function $A_2G_2(t)$, where A_2 is the amplitude, was analyzed by a conventional nonlinear least-squares fitting routine. Satisfactory fitting was obtained with a relative Gaussian distribution $\delta = \Delta\omega_0/\omega_0$ applied to all three frequencies. Nonzero values for δ indicate that the ^{111m}Cd nuclei are located in a distribution of surroundings. An 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.[24]

Results

Macroscopic Dissociation Constants. The macroscopic dissociation constants for the binding of two cadmium ions to the metallo-β-lactamase from *B. cereus* were determined by competitive titrations with the chromophoric chelator Mag-fura-2. In the absence of salt, the values obtained were $K_d' = 12.5$ nM and $K_d'' = 256$ nM. Addition of 0.1 M NaCl had little effect on K_d' (8.3 nM) but produced a significant increase in K_d'' to 5.9 μM.

NMR Spectra. Figure 1 shows the imidazole (N)H proton signals of the enzyme at different [Cd(II)]/[E] ratios. As previously described,[21] when two cadmium ions are present per enzyme, signals are observed from three histidine residues bound to cadmium (residues 88 (BBL 118) and 86 (BBL 116) or 149 (BBL 196) in site 1, and residue 210 (BBL 263) in site 2) and from one buried histidine outside the metal binding sites (residue 28 (BBL 55)). None of these four signals are observed in the apoenzyme.

As cadmium is titrated in to the apoenzyme, from 0 to 1 cadmium equiv, only the H28 imidazole (N)H is observed, but at a different chemical shift from that seen when two cadmium ions are bound to the enzyme. Between 1 and 2 equiv of cadmium, the (N)H resonances of the three cadmium bound imidazoles are observed at the same chemical shift as when two cadmium ions are bound, increasing in intensity with increasing [Cd(II)]/[E] ratio. The H28 (N)H is observed at two

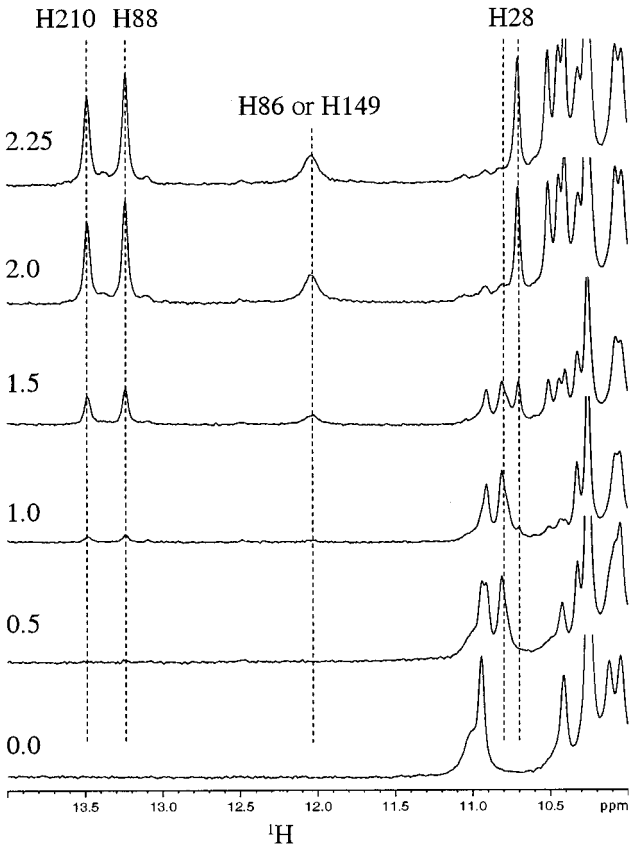

Figure 1. Imidazole (N)H proton NMR signals of the enzyme at different [Cd(II)]/[E] ratios. The [Cd(II)]/[E] ratios are given as numbers to the left of each spectrum. The enzyme concentration was 2 mM. H86, H88, and H149 (BBL H116, H118, and H196) are found at site 1, and H210 (BBL H263) is found at site 2. H28 (BBL 263) is a histidine residue found in the core of the enzyme but not in the active site.

different chemical shifts corresponding to the forms observed between 0 and 1 cadmium equiv and to the binuclear cadmium enzyme, with the relative intensities of these two signals changing with the [Cd(II)]/[E] ratio.

To examine the effects of metal binding on the backbone amide resonances, as an indication of the effects on the protein conformation, ^{1}H−^{15}N HSQC spectra were measured at a series of [Cd(II)]/[E] ratios between 0 and 2.25. Figure 2 shows part of the ^{1}H−^{15}N spectra obtained for [Cd(II)]/[E] ratios of 0, 1.0, and 2.0. The spectra clearly show that many of the cross peaks have different positions in the apoenzyme and in the presence of one and two equiv of cadmium. This demonstrates the existence of a mononuclear cadmium enzyme which is distinct from the apoenzyme and from the species with two cadmiums bound.

Figure 3 shows the ^{113}Cd NMR spectra obtained at different [Cd(II)]/[E] ratios. Two signals, at 142 ppm and at 262 ppm (the assignment of these to cadmium ions bound to sites 1 and 2, respectively, will be reported elsewhere),[32] are observed at a [Cd(II)]/[E] ratio of 2, while for stoichiometries between 0 and 1 only a single, somewhat broader, signal is observed at 176 ppm.

PAC Spectra. In Figure 4 (left panel), the Fourier transforms of the PAC spectra recorded at different [Cd(II)]/[E] ratios are shown, and the results of fitting these spectra are presented in

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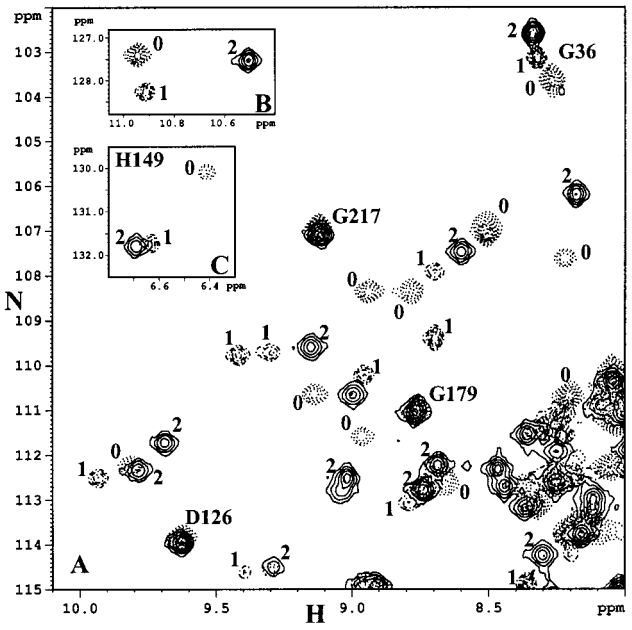

Figure 2. $^{1}H-^{15}N$ HSQC signals for the apoenzyme and the mono- and binuclear cadmium enzymes. (A) $^{1}H-^{15}N$ HSQC of the backbone amide NH for the apoenzyme and the mono- and binuclear enzymes. The enzyme concentration was 2 mM. Dotted lines are for the [Cd(II)]/[E] ratio of 0 (apoenzyme) denoted "0"; dotted−dashed lines are for the [Cd(II)]/[E] ratio of 1 denoted "1"; solid lines are for the [Cd(II)]/[E] ratio of 2 denoted "2". (B) Tryptophan indole NH which exhibits three distinct chemical shifts for [Cd(II)]/[E] ratios of 0, 1, and 2. (C) Active site H149 backbone NH which exhibits three distinct chemical shifts for [Cd(II)]/[E] ratios of 0, 1, and 2.

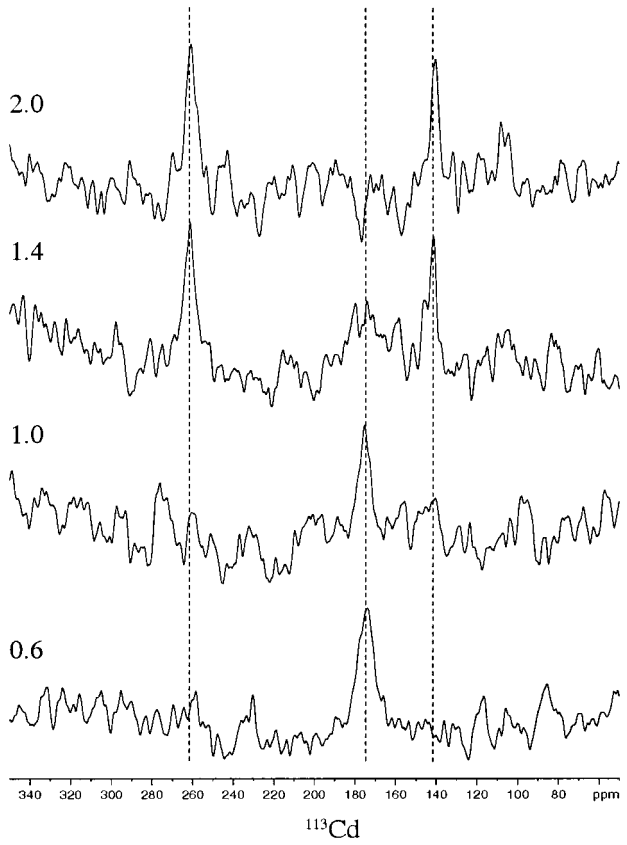

Figure 3. ^{113}Cd 1D NMR spectra at different [Cd(II)]/[E] ratios. [Cd(II)]/[E] ratios are given as numbers to the left. Enzyme concentration was 2 mM.

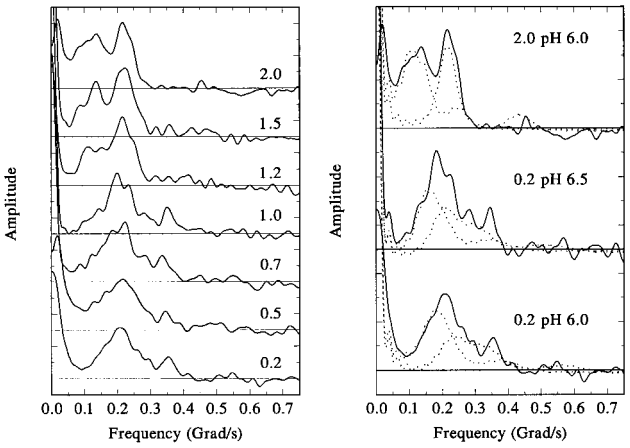

Figure 4. Fourier transform of PAC spectra at different [Cd(II)]/[E] ratios. The [Cd(II)]/[E] ratios are given as numbers in the panel to the left. The sample conditions were 20 mM HEPES, 0.1 M NaCl, 50% W/W sucrose, and pH 6.0, except for [Cd(II)]/[E] ratios of 0.5 and 1.2 where the pH was 5.7 and 6.6, respectively. In the panel to the right, the lower and the upper curves from the left panel together with their theoretical decomposition into two NQIs, shown as the Fourier transform of the fit to the PAC spectra. Also shown in the right panel is the Fourier transform at a [Cd(II)]/[E] ratio of 0.2 at pH 6.5.

Table 1 and Figure 4 (right panel). Noise in the spectra manifests itself as variations around zero (horizontal lines), and the noise level may be estimated by these variations at high frequencies (where no real signal is present). Two almost equally intense NQIs are found at low [Cd(II)]/[E] ratios, and these change into two different, again almost equally intense, NQIs at a [Cd(II)]/[E] ratio of 2, showing that the two cadmium ions interact when they are both bound to the enzyme. Fitting the spectrum at a [Cd(II)]/[E] ratio of 0.2 with one NQI or two NQIs gave reduced χ^2 values of 1.88 and 1.06, respectively (using the first 100 points of $A_2G_2(t)$ which have the best signal-to-noise ratio). Therefore, the data cannot be reproduced by a single NQI. A notable feature is the fact that a very high rate of rotational diffusion is derived, except at pH 6.5, where the rate is comparable to what has been seen before,[5] see Table 1. The dynamic feature (besides rotational diffusion) observed at pH 6.0, but not at pH 6.5, is seen in the right panel of Figure 4 as additional broadening of the peak at zero frequency for a [Cd(II)]/[E] ratio of 0.2. The anomalous rate of rotational diffusion derived at pH 6.0 most likely represents a combined effect of rotational diffusion and some other dynamic process. Thus, fitting with a model for rotational diffusion might not be correct. However, the minimum in the reduced χ^2 was acceptable, and the NQI parameters ω_0 and η were only marginally different between the experiment at pH 6.0 and that at pH 6.5.

Discussion

According to most structure determinations, metallo-β-lactamase from *B. cereus* strain 569/H/9 has two metal ion binding sites, one with three histidine ligands and one solvent ligand (site 1) and the other with a histidine, an aspartic acid, a cysteine, and one or two solvent ligands (site 2).[2−5] The same coordinating amino acids are found in crystal structures of two other metallo-β-lactamases (CcrA and IMP-1)[6−11] and on the basis of sequence alignment in three additional β-lactamases (VIM-1, BlaB, and IND-1).[15] However, there is great variability in the positions and types of solvent ligands and to some extent also in the positions of the coordinating amino acids, indicating a large degree of flexibility at the metal ion binding sites. In

Table 1. Parameters Fitted to the PAC Spectra for [Cd(II)]/[E] Ratios of 0.2 and 2.0 at pH 6.0[a]

NQI_1					
occupancy (%)[b]	η	ω_0 (Grad/s)	δ	τ_R (ns)	[Cd(II)]/[E]
48 ± 8	0.72 ± 0.04	0.118 ± 0.002	0.10 ± 0.02	30 ± 2	0.2
46 ± 10	0.69 ± 0.07	0.105 ± 0.004	0.15 ± 0.02	111 ± 12	0.2 (pH 6.5)
51 ± 3	0.57 ± 0.02	0.079 ± 0.001	0.05 ± 0.01	28 ± 3	2.0
NQI_2					
occupancy (%)[b]	η	ω_0 (Grad/s)	δ[c]	τ_R (ns)[c]	[Cd(II)]/[E]
52 ± 8	0.58 ± 0.06	0.176 ± 0.003	0.10 ± 0.02	30 ± 2	0.2
54 ± 10	0.56 ± 0.06	0.158 ± 0.004	0.15 ± 0.02	111 ± 12	0.2 (pH 6.5)
49 ± 3	1.0 ± 0.2	0.122 ± 0.006	0.05 ± 0.01	28 ± 3	2.0

[a] See Figure 4 and the methods section for experimental conditions. [b] The occupancy gives the amplitude of the signal (NQI_1 or NQI_2) relative to the amplitude of the total signal ($NQI_1 + NQI_2$). [c] δ and τ_R are the same for NQI_1 and NQI_2.

this work, we focus on the dynamics at the metal ion binding sites of the *B. cereus* enzyme. The findings may be relevant for other metallo-β-lactamases as well and may have implications for the catalytic mechanism of the enzymes.

Apo β-Lactamase and Mono- and Binuclear Cadmium β-Lactamase. Figure 2 shows that the $^1H-^{15}N$ HSQC spectra are different for the apoenzyme, for a [Cd(II)]/[E] ratio of less than 1.0, and for a [Cd(II)]/[E] ratio between 1.0 and 2.0. No other signals besides these three species are observed. The behavior of these cross peaks, as the cadmium concentration was increased, was characteristic of slow exchange between three species of enzyme. This demonstrates that the apoenzyme and the mono- and binuclear cadmium enzymes are distinct species. As expected, two ^{113}Cd NMR signals are observed for the binuclear enzyme, one at 142 ppm and one at 262 ppm, see Figure 3. The resonance at 262 ppm may be assigned to cadmium bound to site 2, and the resonance at 142 ppm to cadmium bound to site 1.[32] Similarly, two ^{111m}Cd PAC signals are observed for the binuclear enzyme (with $\omega_0 = 0.079$ Grad/s and $\omega_0 = 0.122$ Grad/s, respectively), see Table 1.

At [Cd(II)]/[E] ratios < 1, only one ^{113}Cd resonance is observed, at 176 ppm, suggesting at first sight that cadmium occupies only one of the two sites under these conditions. However, in contrast, the PAC spectra show two approximately equally populated coordination geometries under these same experimental conditions.

Dynamics of the Metal Ions. The only straightforward explanation for this observation comes from considering the dynamics of metal binding and the different time regimes monitored by the two methods. The effects of exchange between two coordination geometries are qualitatively the same for both techniques. Two geometries in slow exchange will give rise to two signals in both techniques (shielding in NMR and NQI in PAC). In fast exchange only one signal, the weighted average of the two signals characteristic of the two states, is observed for both methods, while for intermediate exchange both techniques give broad signals. However, the time scales to which the two methods are sensitive are quite different: ^{111m}Cd PAC experiments can monitor dynamics in a time regime from about 0.1 to 100 ns, whereas chemical exchange effects in ^{113}Cd NMR, with large chemical shift differences arising from differences in coordination, can typically monitor dynamics from 0.01 to 10 ms.

The most straightforward explanation consistent with both the NMR data and the PAC data at [Cd(II)]/[E] ratios ≤ 1 is that a single cadmium bound to the enzyme jumps between the two binding sites on a time scale between 100 ns and 0.01 ms. This would produce spectra characteristic of slow exchange with PAC, with two distinct NQIs, and characteristic of fast exchange for ^{113}Cd NMR, with a single average line. (Assuming that the ^{113}Cd chemical shift difference between cadmium in site 1 or site 2 in the mononuclear enzyme is similar to that between the two sites in the binuclear enzyme, ~8 kHz, a lifetime of <0.01 ms would lead to a fast exchange average NMR signal.)

This suggestion is supported by the absence of any imidazole (N)H proton signals from the metal coordinating histidines for the mononuclear enzyme, while in the binuclear cadmium enzyme the signals from the imidazole (N)H protons of the cadmium bound histidines are observed for both sites. Solvent exposed imidazole (N)H protons exchange readily with the aqueous solvent and are only observed if they are in a relatively solvent-inaccessible environment. It is thus easy to understand that these protons from the histidines in the metal binding sites are unobservable in the apoenzyme but readily observed in the binuclear cadmium enzyme. It is perhaps more surprising that they are not observable for the mononuclear species. If the single cadmium ion was bound in a site which was stable on the time scale of the NMR experiment, the imidazole (N)H signals should be observed for this site, as they are for the binuclear enzyme. The simplest explanation of the absence of all imidazole N(H) signals from the metal site histidines in the mononuclear cadmium enzyme is, again, that the metal ion jumps between the two sites. If we assume that the imidazole protons are protected from exchange only when a given site is occupied by the metal, but are otherwise labile, then all these protons will exchange rapidly with the H_2O solvent (and hence disappear from the 10−13 ppm region of the spectrum) if the lifetime of the metal in a specific site is less than the chemical shift difference between the N(H) protons and water (~4 kHz). This is consistent with the 100 ns−0.01 ms time scale estimated above from the ^{113}Cd spectra. The fact that the buried His28 imidazole N(H) is observed for the mononuclear cadmium species shows that the dynamic process involved is particular to the imidazoles at the metal ion binding sites, while the fact that the His149 backbone amide signal is observed indicates that it is particular to the side chain and is not propagated to the backbone. This supports the assumption that the dynamics of the histidine (N)H protons is due to exchange with protons in the solvent, rate-limited by exchange of the metal ion.

An alternative possible explanation for the observation of only one ^{113}Cd NMR signal for the mononuclear enzyme would be that metal exchange between the two sites is slow on the ^{113}Cd NMR time scale, but some additional dynamic process, at one of the two sites only, is in intermediate exchange, and the ^{113}Cd signal from this site is therefore too broad to be observed in the spectrum. For example, an additional dynamic process at site 2 might correspond to the apical water molecule jumping on and off the metal ion, a possibility consistent with the conclusions from quantum mechanical studies[33] and with the

presence[4,6−9] and absence[3,5,10,11] of this water molecule in different crystal structures. However, this model does not explain the absence of NH proton signals from all the metal−ligand histidine residues for the mononuclear case, as the site which is not affected by the postulated dynamic process should give rise to observable NH proton signal(s) as well as to an observable ^{113}Cd signal. In addition, this model would imply that three NQIs should be observed in the PAC spectra, two from one site and one from the other. Exchange of the metal between the two sites, by contrast, is consistent with all the NMR and PAC data. If the "jumping metal ion" model is indeed correct, the line at 176 ppm for the mononuclear cadmium enzyme should be the average of the two individual lines from the two metal binding sites which would have been observed in the slow exchange regime. This ^{113}Cd signal disappeared at lower temperature, as would be expected on entering the intermediate exchange regime, but it has not yet been possible to observe the two lines expected to appear in the slow exchange regime at still lower temperature. If we assume that the shifts for the two sites are the same in the mononuclear enzyme as in the binuclear enzyme, a ratio of 70:30 occupancy of site 1:site 2 is deduced from the ^{113}Cd NMR spectrum of the mononuclear cadmium enzyme. Alternatively, assuming a 1:1 occupancy of the two sites as estimated from PAC spectroscopy on the mononuclear cadmium enzyme, and taking the average of the two lines from the binuclear enzyme (142 and 262 ppm), we estimate a weighted average chemical shift of 202 ppm which is somewhat too far downfield. However, it may very well be that the two ^{113}Cd chemical shifts for the mononuclear enzyme are different from those in the binuclear case, given the observation that the two NQIs for the binuclear case are different from those seen in the mononuclear case. Thus, although the exact relative populations of the two sites cannot be determined, they are likely to lie in the range 50:50 to 70:30; that is, cadmium binding to the two sites is essentially equienergetic. In Paul-Soto et al.,[5] NQI_1 was assigned to site 1, and correspondingly NQI_2 to site 2, on the basis of measurements on the Cys168Ala mutant. The NQIs and occupancy for the two sites found in this work are slightly different, probably because of minor differences in the experimental conditions.

A dynamic distribution of the metal ions between the two sites may also explain why signals characteristic for sulfur coordination appear in EXAFS measurements on the zinc containing enzyme[19] and in UV−vis absorption spectroscopy performed on the cobalt containing enzyme[16] at low metal-to-enzyme stoichiometry.

Exchange between the two metal sites in the 100 ns−0.01 ms range requires a relatively flat potential energy surface for the movement of the cadmium ion, with an energy barrier of 33−45 kJ/mol, derived using simple transition state theory. A flat potential energy surface has been found for the zinc−zinc distance in the binuclear zinc enzyme by quantum mechanical calculations,[34] but not yet for the mono-cadmium enzyme.

If the metal ion is exchanging between the two metal ion binding sites, does this occur as an intermolecular or as an intramolecular exchange? The equilibrium constant for cadmium dissociation from the enzyme, when only one metal ion is bound to the enzyme, is (see the results section) $\sim$10 nM $= k_{off}/k_{on}$, where k_{off} is the dissociation rate constant and k_{on} is the association rate constant for cadmium binding to the enzyme. The highest possible k_{on} occurs for diffusion controlled reactions and is on the order of 10^9 M^{-1} s^{-1}. Therefore, the highest possible k_{off} is about 10 s^{-1}, corresponding to dynamics at a time scale of 100 ms. This is much too slow to explain the observed dynamics, which occurs on the microsecond time scale. We conclude that the exchange must occur as an intramolecular process, where the cadmium ion jumps from one metal ion binding site to the other within the same enzyme molecule.

A jumping metal ion may also have implications for the catalytic mechanism of the enzyme. However, it remains to be demonstrated whether the jumping also occurs for the mononuclear zinc enzyme, and when a substrate or an inhibitor is present, or if the substrate or inhibitor stabilizes the binding of the metal ion to one of the two sites.

Anticooperativity in Binding of the Two Cadmium Ions. Another interesting feature of cadmium binding to the enzyme can be deduced from the combination of the PAC spectra and the dissociation constant determinations. Markedly different macroscopic dissociation constants are observed for the binding of one and two cadmium ions (8.3 nM and 5.9 μM, respectively, at 0.1 M NaCl). This may be explained by one of the two sites binding the cadmium ion stronger than the other. In this case, the measured macroscopic dissociation constant is identical to the microscopic dissociation constant for the binding of one cadmium ion. However, in the PAC spectra measured at [Cd(II)]/[E] ratios of less than 1, two NQIs are observed, which have been assigned to the two essentially equally populated metal binding sites.[5] The measured dissociation constant for the binding of one cadmium ion thus corresponds not to the binding to one high affinity site, but to both sites binding the metal ion strongly, with about the same dissociation constant. The higher macroscopic dissociation constant (lower affinity) for the binding of two metal ions must therefore indicate that there is negative cooperativity in the binding of cadmium to the metallo-β-lactamase from *B. cereus* 569/H/9. Additional support for this model is found in the fact that the two NQIs at low [Cd(II)]/[E] ratios are different from the two NQIs at a [Cd(II)]/[E] ratio of 2. This shows that the two cadmium ions interact when they are both bound to the enzyme. Interestingly, the strain 5/B/6 has a much higher value for the macroscopic dissociation constants, particularly the value relevant for low metal-to-enzyme stoichiometry.[5] For the 5/B/6 strain, the amplitudes of the different NQIs as a function of the [Cd]/[E] ratio led to the proposal of positive cooperativity.[5] The data in this work indicate that for the 569/H/9 strain negative cooperativity occurs for the binding of two cadmium ions. An additional difference between the two strains is that, for 5/B/6, there is preferential binding of cadmium ions to site 2. The difference between the enzyme from strain 5/B/6 and that from 569/H/9 is 17 amino acids of which two are close to the active site.[19]

Conclusions

The experiments reported here demonstrate that the combination of NMR and PAC spectroscopy provides unique possibilities for the observation of dynamics at metal ion binding sites in proteins, in the time range from about 0.1 ns to 1 ms. In the case of the *B. cereus* metallo-β-lactamase, this combination of techniques provides clear evidence for a dynamic process at the metal binding sites in the mononuclear cadmium enzyme, which is most probably an intramolecular exchange of the cadmium between the two binding sites on the microsecond time scale.

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