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Positively Cooperative Binding of Zinc Ions to *Bacillus cereus* 569/H/9 β-Lactamase II Suggests that the Binuclear Enzyme Is the Only Relevant Form for Catalysis

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Metallo-β-lactamases catalyze the hydrolysis of most β-lactam antibiotics and hence represent a major clinical concern. While enzymes belonging to subclass B1 have been shown to display maximum activity as dizinc species, the actual metal-to-protein stoichiometry and the affinity for zinc are not clear. We have further investigated the process of metal binding to the β-lactamase II from *Bacillus cereus* 569/H/9 (known as BcII). Zinc binding was monitored using complementary biophysical techniques, including circular dichroism in the far-UV, enzymatic activity measurements, competition with a chromophoric chelator, mass spectrometry, and nuclear magnetic resonance. Most noticeably, mass spectrometry and nuclear magnetic resonance experiments, together with catalytic activity measurements, demonstrate that two zinc ions bind cooperatively to the enzyme active site (with $K_1/K_2 \geq 5$) and, hence, that catalysis is associated with the dizinc enzyme species only. Furthermore, competitive experiments with the chromophoric chelator Mag-Fura-2 indicates $K_2 < 80$ nM. This contrasts with cadmium binding, which is clearly a noncooperative process with the mono form being the only species significantly populated in the presence of 1 molar equivalent of Cd(II). Interestingly, optical measurements reveal that although the apo and dizinc species exhibit undistinguishable tertiary structural organizations, the metal-depleted enzyme shows a significant decrease in its α-helical content, presumably associated with enhanced flexibility.

Keywords: metallo-β-lactamases; cooperativity; NMR; mass spectrometry; circular dichroism

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Abbreviations used: ESI–MS, electrospray ionization mass spectrometry; BcII, β-lactamase II from *Bacillus cereus* 569/H/9; MF, Mag-Fura-2; HSQC, heteronuclear single quantum coherence; HMQC, heteronuclear multiple quantum coherence; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

Introduction

β-Lactamases catalyze the hydrolysis of the β-lactam ring of penicillins and related compounds. Synthesis of one or more of these enzymes constitutes the most common, and often the most efficient, mechanism employed by bacteria to escape the action of β-lactam antibiotics.[1,2] Many β-lactamases have been described and, on the basis of their primary structure, divided into four classes: A, B, C, and D.[3] In enzymes of classes A, C, and D, the hydroxyl group of a serine residue is responsible for the nucleophilic attack on the carbonyl carbon atom of the β-lactam ring, whereas class B β-lactamases rely on the presence of zinc ions [Zn(II)] for their physiological activity. The first of these metallo-β-lactamases (termed BcII) was isolated from an innocuous strain of *Bacillus cereus*[4] and was originally considered as a mere biochemical curiosity. The number of class B enzymes identified has, however, increased steadily over the past 20 years, and they have been found in at least 20 bacterial species, many of which are pathogenic.[5] Metallo-β-lactamase genes carried on transferable genetic elements (both integrons and plasmids) have been detected worldwide.[6,7] In particular, variants of the IMP and VIM enzymes have been identified in numerous pathogenic bacterial strains, and new variants are continuously discovered. A major feature of most metallo-β-lactamases is that they catalyze the hydrolysis of nearly all β-lactam antibiotics used for therapeutic purposes,[8] including the so-called 'β-lactamase-resistant' compounds (e.g., carbapenems, cephamycins, and third-generation cephalosporins), and some mechanism-based inactivators of the class A β-lactamases. The production of a class B enzyme together with one or more serine β-lactamases represents a tremendous advantage for the bacterium, which thereby becomes resistant to nearly all known β-lactam antibiotics, and hence constitutes a major clinical threat.

All known structures of metallo-β-lactamases (11 nonredundant crystal structures are available to date; see Refs. 9 and 10 and references therein) share an αβ/βα sandwich fold, with the catalytic metal center positioned in a groove at the interface between the two domains. The overall structural similarity of metallo-β-lactamases contrasts with their relatively low degree of sequence identity (<20%[11]). On the basis of their primary structures, class B β-lactamases have been divided into three subgroups: B1, B2 and B3.[11,12] Enzymes from each class exhibit specific functional and mechanistic properties.[13,14] In particular, while the B1 and B3 enzymes exhibit maximum activity as dizinc species, the B2 β-lactamases are inhibited upon binding of a second Zn(II) ion.[15] Furthermore, even within subclass B1, to which the majority of metallo-β-lactamases belongs (e.g., BcII, SPM-1, CcrA, BlaB, and the IMP and VIM families), different enzymes have been reported to vary widely in some details of the catalytic mechanism[16–18] and in their affinity for zinc.

The metal-to-protein stoichiometry remains controversial since apparently contradictory findings have been reported. Thus, the X-ray structure of the SPM-1 β-lactamase[19] has been determined with one Zn(II) only, bound to the tri-histidine site (known as the histidine site), which is strictly conserved among B1 enzymes (His116, His118, and His196). In contrast, the crystal structures of CcrA[20], IMP-1,[21] and BlaB[22] β-lactamases showed a second Zn(II) bound to the other site (known as the cysteine site), which is also strictly conserved in subclass B1 (Asp120, Cys221, and His263). Structures of the BcII[23–25] and VIM-2[26] enzymes indicated an intermediate character, since they were reported with either the histidine site only or both sites occupied. The mononuclear forms of SPM-1 and BcII are catalytically active, but the dizinc form of the latter has been found to be substantially more active (upon binding of the second zinc ion, a 2-fold increase of the k_{cat} value is observed with benzylpenicillin as substrate[27,28]). Although it has been proposed that under physiological conditions, all metallo-β-lactamases work as monozinc enzymes,[29] the actual metal-to-protein stoichiometry remains an open question.[30] Large variations in zinc affinity among the B1 enzymes have, however, been observed *in vitro* and no satisfactory explanation could be proposed so far. Even more obscure is the wide range of values reported for the dissociation constants (K_1 and K_2, see Materials and Methods) measured with BcII. Thus, although all studies are consistent with a small value for the K_1/K_2 ratio ($\sim 10^{-3}$), indicating negative cooperativity for binding the second zinc ion, very different values have been reported for the two dissociation constants, ranging from 0.6 nM to 120 μM and from 1.5 μM to 24 mM, for K_1 and K_2, respectively.[28,29,31–33] These apparent inconsistencies are probably due, at least in part, to different experimental conditions (e.g., pH, ionic strength, and enzyme concentration). A further difficulty might also arise from the utilization of slightly different BcII enzymes, produced by two different *B. cereus* strains, namely, 569/H/9 and 5/B/6.[34] Although the two β-lactamases differ only by 17 substitutions, which are all outside the catalytic center, it cannot be totally excluded that these mutations influence the affinity of the enzyme for exogenous zinc ions.

In this work, we have further characterized the process of zinc binding to BcII (569/H/9). Comparison of the results of physical measurements, notably mass spectrometry (MS) and nuclear magnetic resonance (NMR), with measurements of enzymatic activity shows clearly that two zinc ions bind cooperatively to BcII (569/H/9) and that the dizinc enzyme is the only one relevant to catalysis. Cadmium binding, being clearly noncooperative, provides an interesting contrast.

Results and Discussion

Determination of the catalytic parameters with benzylpenicillin as substrate (in 10 mM Hepes,

pH 7.5, 30 °C) gave $k_{cat}=1000\pm30$ s^{-1}, $K_m=450\pm30$ μM, and $k_{cat}/K_m=2.2\pm0.3\times10^6$ $M^{-1}\cdot s^{-1}$. These values are in reasonable agreement with those obtained by Paul-Soto *et al.*[27] (i.e., 310 s^{-1}, 440 μM, and 0.7 10^6 $M^{-1}\cdot s^{-1}$, respectively, at 25 °C) and Badarau and Page[35] (950 s^{-1}, 1100 μM, and 0.87 10^6 $M^{-1}\cdot s^{-1}$, respectively, at 30 °C), under similar conditions. They suggest full activity of the enzyme preparation.

Structural comparison of BcII apo and dizinc

Metal-free BcII β-lactamase (i.e., apoenzyme) was prepared as described in Materials and Methods. The enzyme (10 μM) was totally inactive towards 1 mM benzylpenicillin (extinction coefficient difference of -800 $M^{-1}\cdot cm^{-1}$ at 235 nm; $\Delta A_{235\ nm}<0.005$ in a 1-min period). Furthermore, the electrospray ionization (ESI) mass spectrum of the apoenzyme (see below), sprayed under non-denaturing conditions, shows one major species corresponding to a molecular mass of 24,960±1 Da, as expected from the amino acid sequence of the enzyme (calculated $M_r=24{,}960$), and no trace of the mono or dizinc species. These data validate the preparation protocol of the apoenzyme.

Possible conformational changes resulting from Zn(II) removal were estimated by fluorescence and circular dichroism (CD) spectroscopy. Near-UV CD (250–310 nm) and fluorescence emission spectra (data not shown) indicated only minor differences in the tertiary structural organization of the enzyme, in the absence and presence of Zn(II). The far-UV CD spectra of free and metal-bound BcII are displayed in Fig. 1. A qualitative analysis indicates that both spectra are dominated by the contribution of α-helices, with negative bands at ~222 and ~208 nm, but comparison of the two spectra shows a clear difference in their relative magnitudes. Deconvolution of the two spectra (Table 1) using the program CDSSTR (see Materials and Methods) reveals a significant increase in helical content of the protein in the presence of zinc. This occurs at the expense of disordered structure, whereas little or no change in β-strands and turns content is observed. The difference between the apo- and holoenzymes is confirmed by NMR heteronuclear single quantum coherence (HSQC) experiments (see below), which reveal significant chemical shift perturbations and signal broadening in the spectra of the zinc-depleted form and, thus, indicate possible structural changes and an increase in the protein flexibility. These observations contrast with the small changes restricted to the active-site environment, observed in the 1.85-Å-resolution X-ray structure of the apoprotein.[24] These data, however, were obtained by washing the holoprotein crystals with a buffer containing 1 mM ethylenediaminetetraacetic acid (EDTA) and no zinc. Under these conditions, the clear conformational change that we observe in solution might not take place. Our data indicate that the metal ions in the BcII β-lactamase not only have a catalytic role but also have a structural role. These findings are in good agreement with the large decrease in enzyme stability observed after zinc removal (O.J. and A.M., unpublished results).

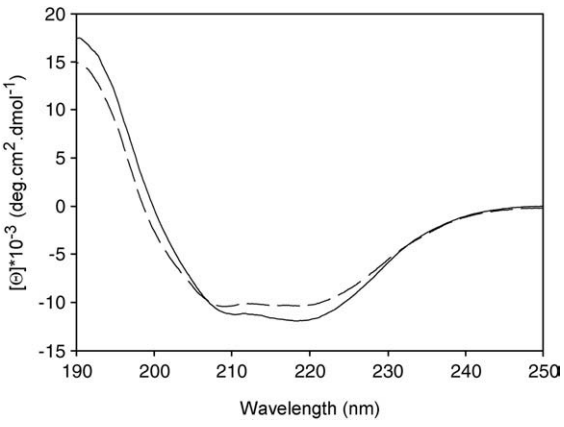

Fig. 1. Far-UV CD spectra of apoBcII and holoBcII β-lactamases. Data were obtained at 25 °C, in H_2O, in the absence (broken line) and presence (continuous line) of 100 μM $ZnSO_4$. The protein concentration was 15 μM. CDSSTR-calculated helical contents estimated from these spectra are ~20% and ~30% in the absence and in the presence of Zn(II), respectively.

Table 1. Secondary-structure content of metal-free (apo) and metal-bound (holo) BcII β-lactamases, as estimated from the analysis of the far-UV CD spectra shown in Fig. 1

	Helix (%)	Strand (%)	Turn (%)	Disordered (%)	Total (%)
Holoenzyme	30±4	20±2	19±1	31±2	100
Apoenzyme	21±4	23±3	21±3	36±3	101

Data were obtained by averaging results obtained after deconvolution of two series of CD spectra and by using protein data sets n° 4 and n° 7 in DICHROWEB. These data are in reasonable agreement with the X-ray data available for zinc-bound BcII (i.e., 23–26% α-helix and 27–30% β-strand, calculated in the Research Collaboratory for Structural Bioinformatics Protein Data Bank, using the 1bvt Protein Data Bank structure).[24]

Zinc binding to BcII β-lactamase: Catalytic activity measurements

Binding of Zn(II) to the metal-free BcII β-lactamase was monitored by measuring the recovery of enzymatic activity upon addition of $ZnSO_4$. In order to minimize the possible effect of residual exogenic Zn(II) in the metal-free buffer [<0.1 μM Zn(II)[15,27,36,37]], we selected a rather "poor" substrate of the enzyme, that is, cephalexin. The low turnover number of the enzyme (we measured $k_{cat}=8.1\pm0.3$ s^{-1}, $K_m=103\pm6$ μM, and $k_{cat}/K_m=78{,}500\pm6000$ $M^{-1}\cdot s^{-1}$ at 30 °C, in 10 mM Hepes, pH 7.5, which is consistent with the values obtained by Badarau and Page[35] under slightly different conditions: 7.3 s^{-1}, 300 μM, and 24,000 $M^{-1}\cdot s^{-1}$, respectively) allowed us to use a micromolar enzyme

concentration (6.2 μM) about 2 orders of magnitude above the expected residual Zn(II) concentration.

Initial rate values for the hydrolysis of cephalexin determined in the presence of various Zn(II) concentrations (0–25 μM) are reported in Fig. 2. A linear increase in specific activity is observed upon the addition of 0 to 2 equivalents of Zn(II), and then a plateau is reached, suggesting complete saturation of the two Zn(II) binding sites. Note that addition of zinc to the apoenzyme restored ~100% of the initial enzyme activity. Very similar results (not shown) were obtained in 20 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes) and 100 mM NaCl, pH 6.4, that is, under conditions used for NMR experiments.

These results are compatible with at least three different situations: (i) Noncooperative binding of the two Zn(II) (i.e., the two zinc binding sites have the same intrinsic affinity, and the affinity of one site is independent of whether or not the other site is occupied), with the activity of the monozinc species being ca 50% that of the dizinc one.[27] Alternatively, Zn(II) binding might be a cooperative process, cooperativity being either positive or negative. (ii) Positive cooperativity; in this case, the monozinc species is not significantly populated at any [Zn(II)]/[E] ratio and the increase in catalytic activity observed upon zinc addition (Fig. 2) corresponds to the formation of the fully active dizinc form of the enzyme. (iii) Negative cooperativity; here, the monozinc species forms first and reaches 100% at [Zn(II)]/[E] = 1, and the dizinc species only appears upon further addition of zinc. Note that models (i) and (iii) are only compatible with the data in Fig. 2 if the monozinc species exhibits 50% of the activity of the dizinc form. In order to discriminate between these possible scenarios and to further understand the mechanism of Zn(II) binding to the BcII β-lactamase, we used MS and NMR techniques to monitor Zn(II) binding to the enzyme.

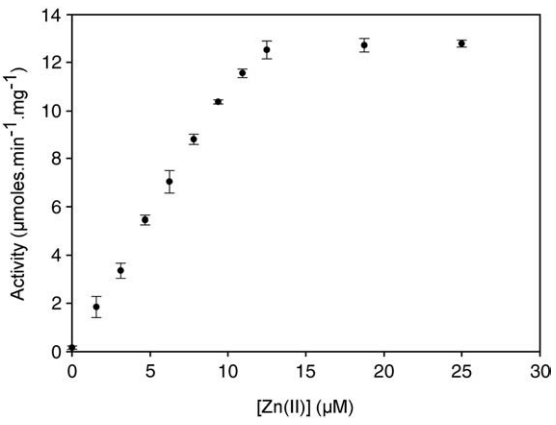

Fig. 2. Zinc titration of apoBcII enzyme, as monitored by catalytic activity. The enzyme concentration was 6.2 μM, and the effect of Zn(II) concentration [0–25 μM, i.e., 0 to 4 Zn(II) equivalents] on the hydrolysis of 100 μM cephalexin was measured in 10 mM Hepes, pH 7.5, at 30 °C. Initial rate (v_0) values were obtained by following substrate depletion on a 1-min time scale.

Zinc binding to BcII β-lactamase: MS

Samples of apoBcII (10 μM), containing different zinc concentrations (from 0 to 22.2 μM), were analyzed under non-denaturing conditions. Spectra of samples obtained at different zinc-to-enzyme ratios (i.e., 0, 0.89, and 2) are shown in Fig. 3a. (Note that these experiments were reproducible and that similar results have recently been reported by others.[38])

Three well-defined species with different masses can be detected at different [Zn(II)]/[E] ratios. One corresponds to the apoenzyme (M_r = 24,961 ± 2) and is the only species significantly populated in the absence of Zn(II). Another peak, which is 128 Da higher in mass, corresponds to the dizinc enzyme (M_r = 25,089 ± 2) and predominates at [Zn(II)]/[E] ≥ 2. The third peak, which shows intermediate mass (M_r = 25,025 ± 2), corresponds to a monozinc species. The mass differences, that is, 64 and 128 between the apoenzyme and the monozinc enzyme and between the mono and dizinc enzymes, respectively, suggest the loss of two protons for each bound Zn(II) ion.[39] At zinc ion-to-enzyme ratios between 0 and 2, the three species can be observed concomitantly in the spectra. Their relative abundances are reported in Fig. 3b. It is worth noting here that a qualitative analysis of these MS data is valid only if the response factors of the various species are similar. Although the monozinc species is observed throughout the whole titration process, its relative abundance always remains below 20%. These data suggest positive cooperativity upon Zn(II) binding, that is, binding at one site increases the affinity at the other one. Interestingly, combination of Eqs. (2) and (3) allows calculation of the K_1/K_2 ratio (see Materials and Methods):

$$K_1/K_2 = [E] \cdot [EM_2]/[EM]^2 \qquad (1)$$

MS data obtained at different [Zn(II)]/[E] ratios give values for [E], [EM], and [EM_2], and hence, a series of values can be calculated for the K_1/K_2 ratio. Averaging those values obtained at 0.67 ≤ [Zn(II)]/[E] ≤ 1.78 leads to K_1/K_2 = 4.6 ± 0.9, which is consistent with positively cooperative binding of Zn(II) to the two metal sites of the enzyme.

In order to validate the use of MS for monitoring zinc binding to the two metal sites of BcII, we repeated the experiment under identical conditions, using cadmium as ligand. With this latter metal, the combination of NMR and PAC spectroscopies[40] revealed that binding is a noncooperative process, with successive population of mononuclear and binuclear cadmium enzyme. The results of the titration experiment are shown in Fig. 3c. The occurrence of 100% of the mononuclear enzyme in the presence of 1 equivalent of cadmium and the relative quantities of the three distinct species observed at different cadmium ion-to-enzyme ratios are in good agreement with previous experiments.[40] This confirms MS as a suitable technique for distinguishing

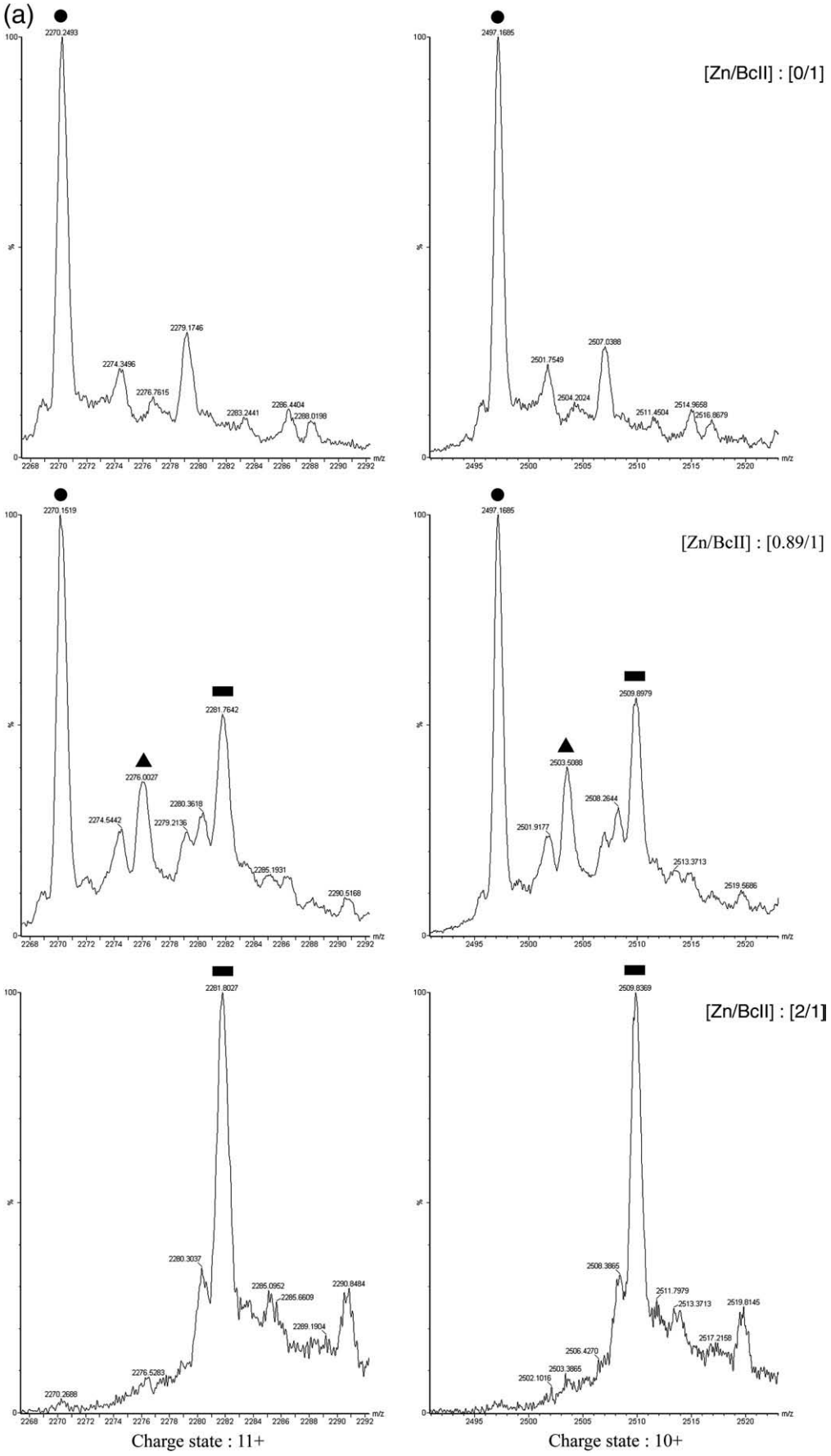

Fig. 3 (*legend on next page*)

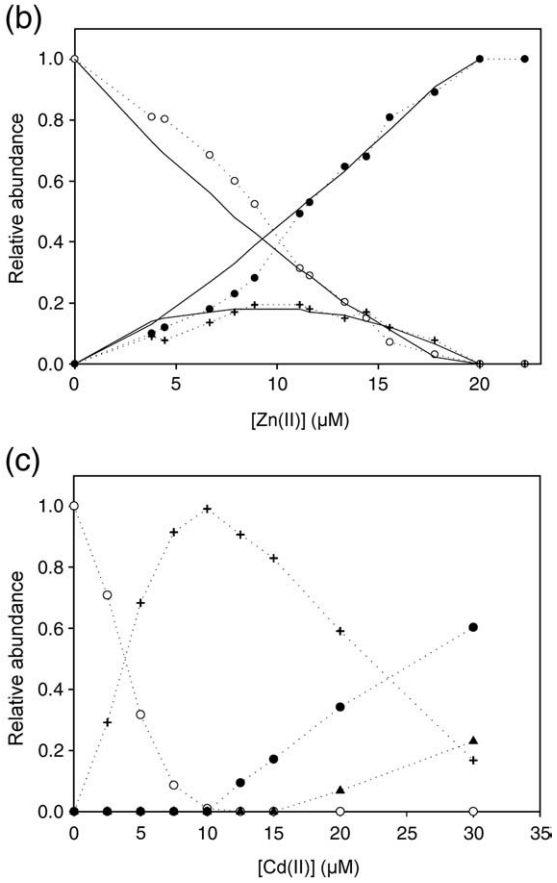

Fig. 3. Zinc titration of apoBcII enzyme, as monitored by MS. (a) ESI mass spectra of the 10+ (right) and 11+ (left) charge states of BcII β-lactamase in the presence of increasing Zn(II)-to-enzyme ratio. Three well-defined populations are detected, which correspond to the apoenzyme (labeled with ●; 10+ state: 2497.2 m/z; 11+ state: 2270.2 m/z; $M_r = 24{,}961 \pm 2$), to the monozinc enzyme (labeled with ▲; 10+ state: 2503.5 m/z; 11+ state: 2276.0 m/z; $M_r = 25{,}025 \pm 2$), and to the dizinc enzyme (labeled with ▬; 10+ state: 2509.9 m/z; 11+ state: 2281.8 m/z; $M_r = 25{,}089 \pm 2$). The protein concentration was 0.25 mg·mL^{-1} (10 μM) in 10 mM ammonium acetate, pH 7. (b) Titration of the enzyme (10 μM) as a function of zinc concentration, as monitored by ESI–MS. The fraction (i.e., relative abundance) of apo- (open circles), monozinc (crosses), and dizinc (filled circles) enzyme is shown. Dotted lines are there to guide the eye only, whereas continuous lines are constructed from the analytical solutions for the relative abundances of the three enzyme species, using Eqs. (2) and (3) with $E_0 = 9.3$ μM, $K_1 = 0.1$ μM, and $K_2 = 0.02$ μM (see the text). (c) Titration of the enzyme (10 μM) as a function of cadmium concentration, as monitored by ESI–MS. The fraction (relative abundance) of apo- (open circles), monocadmium (crosses), dicadmium (filled circles), and tricadmium (triangles) enzyme is shown. Dotted lines are there to guide the eye only.

between the different nuclear forms of the BcII β-lactamase. The occurrence of nonspecific cadmium binding at [Cd(II)]/[BcII] ≥ 2 (Fig. 3c), which was not reported before and is not observed in NMR experiments (see below), does not seem to perturb significantly the titration of the two enzyme binding sites. Nonspecific Cd(II) binding has been observed with other proteins (e.g., hexokinase[41]) and might be favored here in the conditions of the MS experiments.

Zinc binding to BcII β-lactamase: NMR

^{1}H–^{15}N NMR experiments allow observation of the enzyme at the level of individual backbone NH groups and histidine imidazole NH and N(C)H groups. Both cadmium and zinc have been used to titrate the apoenzyme.

Upon titration with cadmium, the backbone NH signals characteristic of the apoenzyme gradually disappear and are replaced by a new set of signals for which the maximum intensity is reached at a [Cd(II)]/[BcII] ratio of 1 (Fig. 4). Using cadmium NMR and PAC spectroscopies, this species was identified as the monocadmium enzyme.[40] As the [Cd(II)]/[E] ratio is increased beyond 1, the signals from the monocadmium enzyme decrease and a third set of signals appears, corresponding to the dicadmium species; this is the only species observed at and beyond 2 cadmium equivalents. Thus, over the whole titration experiment, three independent species are observed, corresponding to the apoenzyme, the monocadmium, and the dicadmium enzymes. The apo- and dicadmium enzymes are never observed simultaneously, indicating noncooperative binding of the metal ions, in good agreement with the results obtained by MS.

In contrast, upon zinc titration (Fig. 5), signals characteristic of the dizinc enzyme are readily observed from the beginning of zinc addition. At concentrations of zinc in the range $0.2 \leq [Zn(II)]/[E] \leq 1.8$, both the apo- and the dizinc enzymes are present but no other species can be detected. Apoenzyme signals remain detectable until addition of 2 zinc equivalents (i.e., [Zn(II)]/[E] = 2). No signals indicative of the presence of a monozinc intermediate species are detected along the whole titration experiment (Fig. 5). This observation contrasts with the observation of up to ~20% of monozinc species at [Zn(II)]/[E] ≈ 1 in the MS experiments. However, it should be noted that the limit of detection of the different enzyme species in NMR experiments is ca 10%, and it is possible that the amount of monozinc species calculated in MS experiments is slightly overestimated. Nonetheless, both techniques indicate that cadmium binding and zinc binding lead to very different situations, with the mononuclear enzyme being observed as a major species with cadmium only.

Selective observation of the imidazole NH resonances of the seven histidines of BcII confirms the differences between cadmium and zinc binding. In the apoenzyme, with the exception of His137, imidazoles give rise to broad NH signals due to conformational exchange and are poorly observed in the spectrum (Fig. 6a). On the contrary, dizinc or dicadmium enzyme imidazoles are very well defined and easily detected (Figs. 6b and 7b).

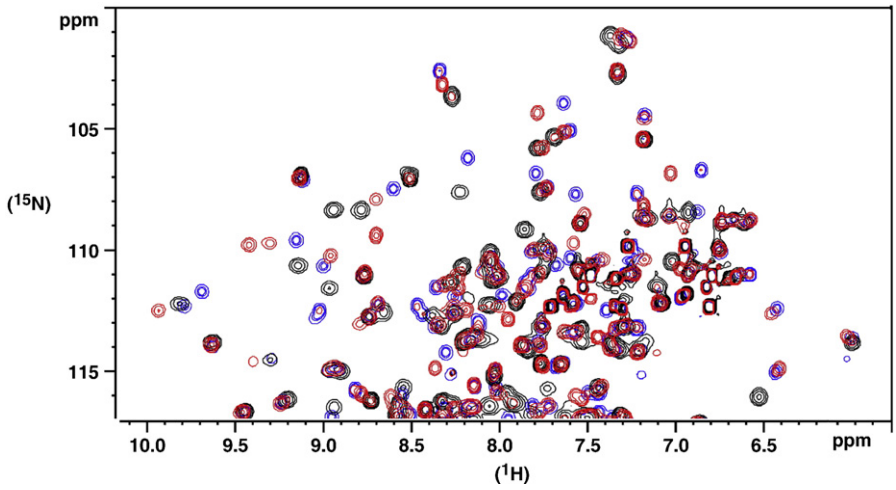

Fig. 4. $^{1}H–^{15}N$-HSQC spectra of BcII β-lactamase in the presence of various amount of cadmium. The enzyme concentration was 1 mM. Signals for [Cd(II)]/[E] ratios of 0, 0.9, and 2 are shown in black, red, and blue, respectively. Three sets of signals are clearly distinguishable, corresponding to the apo- (black), monocadmium (red), and dicadmium (blue) enzyme species.

In the monocadmium species (Fig. 6a), the cadmium is in fast exchange between the two metal binding sites,[40] and hence, the signals of the four binding site imidazoles are broad and weak, as with the apoenzyme. The His55 imidazole, however, which is buried in the core of the native protein, is clearly observed, indicating that this side chain is not in chemical or conformational exchange and, therefore, that this region of the protein is well defined in the monocadmium enzyme. This contrasts with the apoenzyme (Fig. 6a) in which only His137 is observed. In the native enzyme, the protonated imidazole of His137 is involved in a salt bridge with two aspartyl residues, which is probably maintained in the apoenzyme. Dicadmium enzyme imidazole signals, which are unambiguously identified by the nitrogen–cadmium scalar coupling (100–200 Hz), are observed only at [Cd(II)]/[E] ratios greater than 1 (Fig. 6b).

In contrast, zinc titration leads to the observation of dizinc enzyme imidazole signals upon addition of only 0.2 molar equivalent of zinc to the apoenzyme (Fig. 7). These signals gradually increase in intensity while the apoenzyme signals decrease concomitantly as the zinc concentration is raised. No species other than the apo- or dizinc enzyme can be detected during the whole titration. The concentration of a monozinc species can be estimated to be less than 10% of total enzyme concentration at any point during the titration.

This last series of NMR experiments, where the imidazole groups are specifically monitored upon metal titration of the apoenzyme, reinforce the view that a mononuclear species is highly populated in the presence of Cd(II) (up to 100% in the presence of an equimolar concentration of metal), whereas the corresponding species is not detected in the presence of Zn(II) (less than 10% at any zinc concentration).

Zinc binding to BcII: Far-UV CD measurements

Binding of Zn(II) to the apoenzyme was monitored by CD measurements at 219 and 209 nm. At these wavelengths, both the metal-free and metal-bound enzymes display maxima in negative ellipticity (Fig. 1), which are characteristic of α-helical structures. Significant differences in magnitude are seen between the two forms of the enzyme, and these are used here to monitor binding of Zn(II) to the metal-free β-lactamase. At both wavelengths, however, changes in amplitude for the intensity of the CD signal upon Zn(II) addition are weak, and we calculated the ratio of the signal values measured at 219 and 209 nm ($\Theta_{219}/\Theta_{209}$), in order to increase the signal-to-noise ratio in the titration experiment. The result of the titration of metal-free BcII β-lactamase (15 μM) by Zn(II) (0–75 μM) is shown in Fig. 8. The CD parameter ($\Theta_{219}/\Theta_{209}$) value increases linearly between 0 and 30 μM of Zn(II) (i.e., [Zn(II)]/[E] = 0–2), until a plateau is reached at [Zn(II)] > 30 μM (i.e., [Zn(II)]/[E] > 2). A qualitative analysis of these data suggests that no monozinc species is formed significantly and that the Zn(II) ions have high affinities (i.e., both dissociation constants <1 μM) for the two binding sites of the enzyme (i.e., histidine and cysteine sites). No evidence is found for the presence of a monozinc form, which, if present at any stage of the titration, should display a CD spectrum indistinguishable from that of the dizinc species.

Zinc binding to BcII: Competition experiments with Mag-Fura-2 chelator

We used the chromophoric chelator Mag-Fura-2 (MF) to estimate the affinity of the metal-free BcII β-lactamase for Zn(II) ions. A competition experiment was performed, in which BcII apoenzyme was

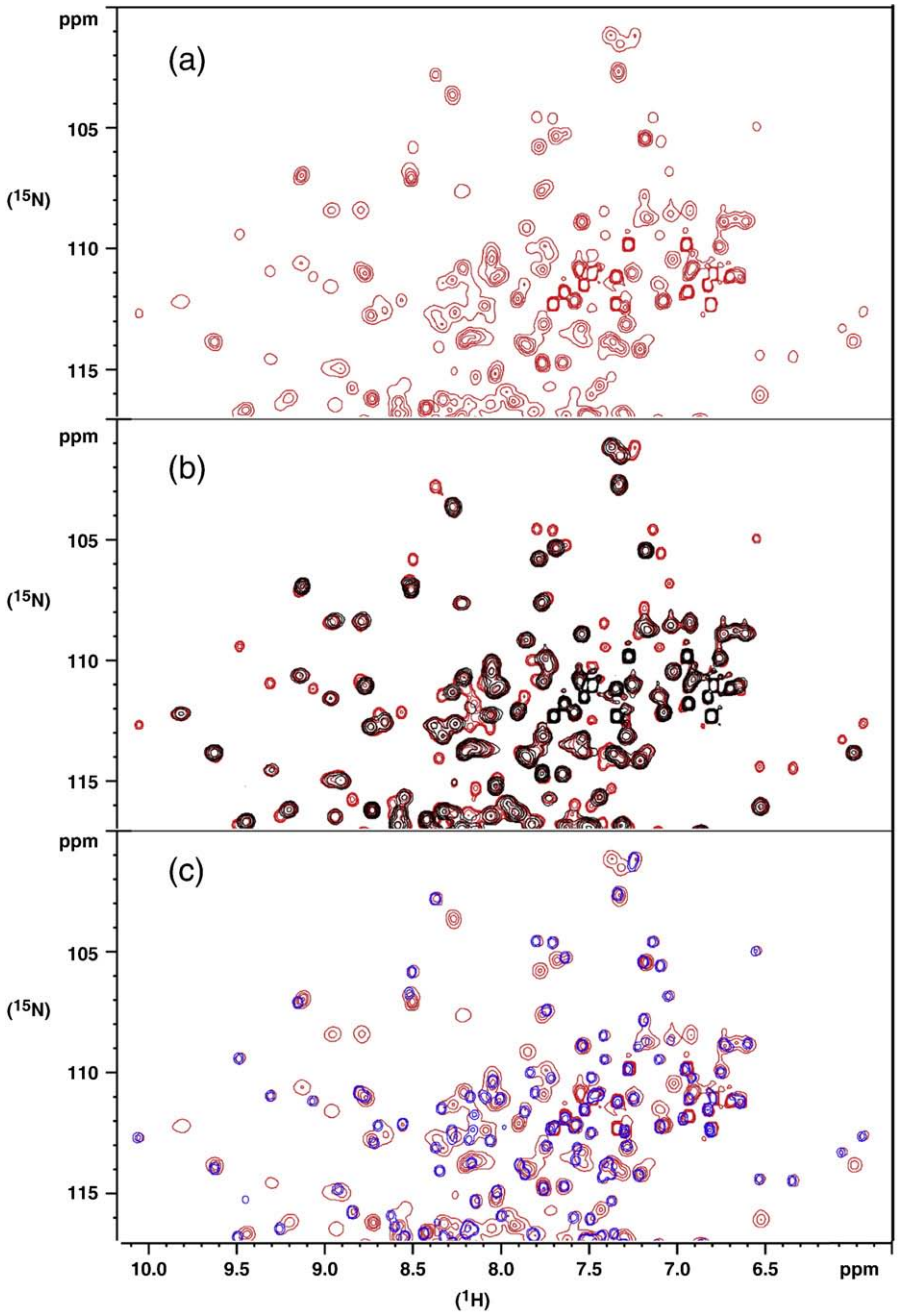

Fig. 5. ^{1}H–^{15}N-HSQC spectra of BcII β-lactamase in the presence of various amount of zinc. The enzyme concentration was 1 mM. (a) HSQC spectrum of the BcII-enzyme with 0. 5 molar equivalent of Zn(II). (b) Superposition of two HSQC spectra: 0. 5 molar equivalent of Zn(II) (red) and apoenzyme (black). All apoenzyme signals (black) are observed in the 0. 5 molar equivalent of Zn(II) spectrum (red). (c) Superposition of two HSQC spectra: 0. 5 molar equivalent of Zn(II) (red) and 2. 0 molar equivalents of Zn(II) (blue). All dizinc enzyme signals (blue) are observed in the 0.5 zinc molar equivalent spectrum (red). At 0. 5 molar equivalent of Zn(II), only two sets of signals are observed corresponding to the apo- (black) and dizinc (blue) enzymes. At any concentration of zinc, no additional peaks are seen in the spectrum, which thus appears as a linear combination of the spectra of the apo- and the dizinc proteins.

added stepwise to a 12 μM solution of MF complexed with Zn(II) (1:1 zinc/indicator). The titration data for the Zn(II)–MF complex with apoBcII are shown in Fig. 9a. The spectra show a typical isosbestic point at 342 nm[33] and maxima at ~365 and ~325 nm for the metal-free compound and the 1:1 zinc complex, respectively. Figure 9b represents the increase in absorbance monitored at 365 nm upon addition of the apoenzyme. A linear absorbance change can be observed between 0 and 6 μM apoBcII, indicating uptake of two zinc ions by one enzyme molecule at the end point of the titration. Furthermore, the data reveal that the enzyme has a higher affinity for Zn(II) than MF. In a previous study, de Seny *et al.* measured a value of ~80 nM for the dissociation constant for the Zn(II)–

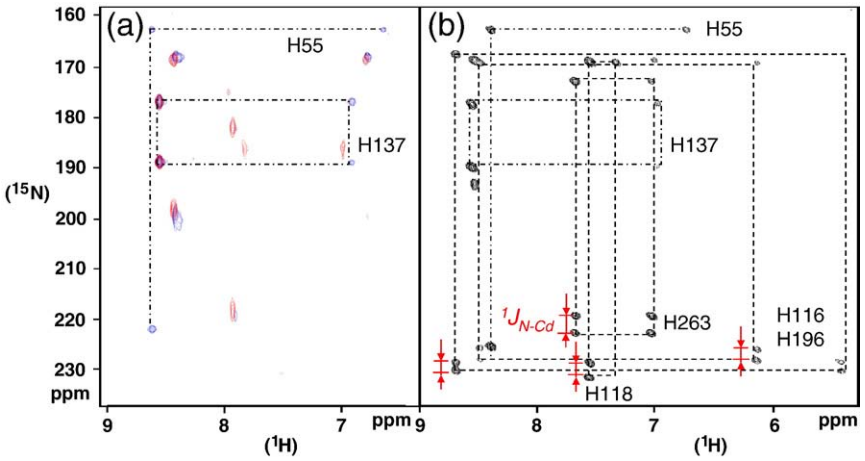

Fig. 6. Long-range ^{1}H–^{15}N HMQC for selective His imidazole observation during cadmium titration. (a) Superposition of apoenzyme (red) and 1. 0 molar equivalent of Cd(II) (blue). Imidazole (H137) is protonated (imidazolium) in the apoenzyme and is not affected by cadmium binding. Imidazole (H55) buried in the core of the protein is neutral and is observed for the monocadmium and dicadmium enzymes but not for the apoenzyme. (b) T wo molar equivalents of Cd(II). Nitrogen-cadmium scalar coupling ($^1J_{N\text{-}Cd}$) is observed for the four Cd(II)-bound imidazoles and is indicated by double red arrows. His imidazoles are labeled (H116, H118, H196, and H263 are the metal binding His).

MF complex.[33] Since this was obtained under very similar experimental conditions, our data indicate that at least the second zinc binding site of the enzyme displays a dissociation constant value lower than 80 nM. This is in agreement with the value of 30±5 nM recently reported by Badarau and Page, which was determined by isothermal titration calorimetry.[37]

Computational analysis of the data

Combination of our experimental data with Eqs. (2) and (3) does not allow direct calculation of the two dissociation constant (K_1 and K_2) values. We were able, however, to simulate the experimental MS data by calculation of the relative proportion of the three enzyme species (i.e., apoBcII, monoBcII, and holoBcII) in the presence of various Zn(II) concentrations, for a number of K_1 and K_2 values. These were restricted, on the basis of experimental evidences (see above), to $K_1/K_2 = 5$ and to $K_2 < 80$ nM. For K_1 values comprised between 1 and 400 nM (and thus 0.2 nM $\leq K_2 \leq$ 80 nM), all simulations gave very similar results, which are quite consistent with the MS data. In all cases, however, comparison of the calculated proportions (i.e., relative abundances) of the three enzyme forms with the actual experimental data revealed an excess

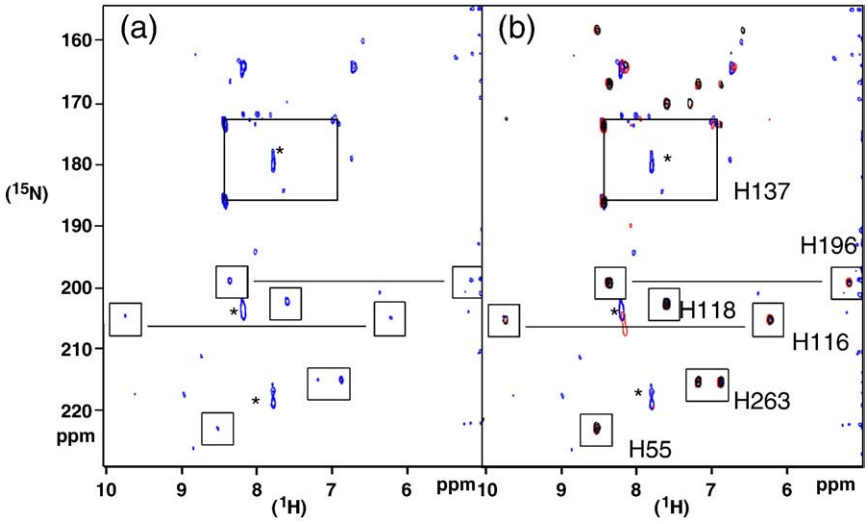

Fig. 7. Long-range ^{1}H–^{15}N HMQC for selective His imidazole observation during zinc titration. (a) 0.2 molar equivalent of Zn(II). Signals characteristic of the dizinc enzyme are indicated with boxes. (b) Superposition of 0.2 (blue), 1.0 (red), and 2.0 (black) molar equivalents of Zn(II). His imidazoles are labeled (residues 116, 118, 196, and 263 are the metal binding histidines). Signals labeled with an asterisk are characteristic of the apoenzyme and are still present at 1 zinc equivalent [in red in (b)]. Signals corresponding to the dizinc enzyme are observed from 0. 2 zinc molar equivalents. No other signal set than those corresponding to the apo- and dizinc enzyme species is observed throughout the whole titration.

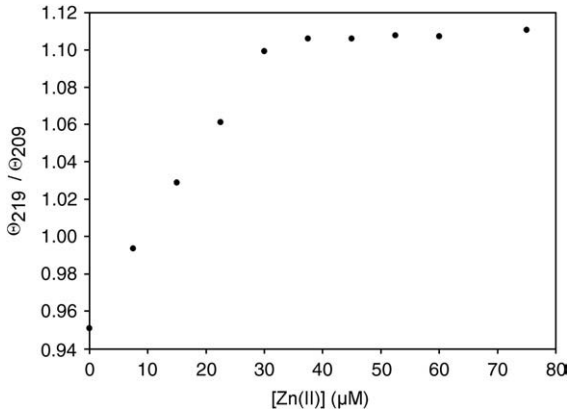

Fig. 8. Zinc titration of apoBcII enzyme, as monitored by far-UV CD spectroscopy. Data were recorded at 25 °C, and the enzyme concentration was 15 μM in 10 mM Hepes and 300 mM NaCl, pH 7.5, containing 0 to 75 μM Zn(II) [i.e., 0 to 5 Zn(II) equivalents].

in free enzyme (and thus a lack of bound enzyme) at [Zn(II)]/[E]>1. This is most probably due to a slight overestimation of the active enzyme concentration (5–10%), and this can be accounted for by reducing the enzyme concentration in the calculation (Fig. 3b).

Conclusions

A homogeneous (>98%) solution of recombinant BcII β-lactamase was produced, which displays full activity towards benzylpenicillin. The enzyme is identical with the original *B. cereus* 569/H/9 β-lactamase, with catalytic parameter values similar to those previously reported. An efficient procedure was developed to produce the metal-depleted form of the enzyme (apoBcII), which is catalytically inactive and displays the expected molecular mass of 24,960 Da.

Following addition of 2 molar equivalents of zinc ions, both the catalytic activity and the far-UV CD spectrum of the original dizinc β-lactamase could be restored. Both MS and NMR experiments demonstrate positively cooperative uptake of two zinc ions by the apoenzyme. Although up to 20% of monozinc species is observed during the titration of the enzyme monitored by MS measurements, no evidence for the population of such species is obtained from NMR experiments. This apparent discrepancy might be due to the different sensitivities of the two methods and perhaps to the experimental conditions inherent to ESI–MS experiments as well. With both techniques, however, positive cooperativity is observed with $K_1/K_2 \geq 5$. Furthermore, competition experiments with the chromophoric chelator MF indicates that $K_2 < 80$ nM. On this basis, simulations of the relative abundances of the three enzyme species (i.e., apo-, mono-, and dizinc enzymes) performed at various [E]/[Zn(II)] ratios (e.g., with $K_1 = 100$ nM and $K_2 = 20$ nM, see Fig. 3b) are in good agreement with the experimental data. By contrast, we find, in agreement with previous observations,[27,40] that Cd(II) binding to BcII is a noncooperative process and is characterized by the occurrence of 100% of the mononuclear cadmium β-lactamase in the presence of 1 molar equivalent of Cd(II).

Thus, in our hands, binding of zinc ions to the BcII β-lactamase occurs with positive cooperativity and the dizinc form appears to be the only relevant species for catalysis. This is in agreement with the recent work by Badarau and Page, which has shown that at low zinc ion concentrations, a zinc ion dissociates from the enzyme active site during turnover, resulting in a mononuclear enzyme species with very little or no activity, which requires the reassociation of a zinc ion for hydrolysis.[37] Badarau and Page used isothermal titration calorimetry to monitor zinc binding to apoBcII in 25 mM Mops, pH 7.06, at 30 °C.[37] Their data show only one binding event, with a stoichiometry of ~2, and allow calculation of 30±5 nM for the dissociation constant value, which is consistent with our hypothesis of positively cooperative binding of zinc ions. This behavior is similar to that observed with the homologous subclass B1 β-lactamase from *Bacillus fragilis* (CcrA[42]) and reconciles the zinc

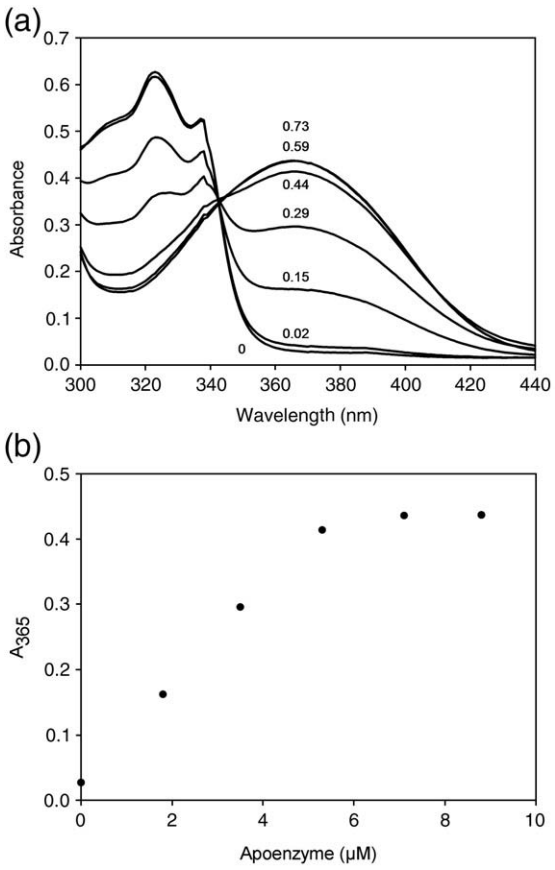

Fig. 9. Competition for Zn(II) binding between BcII β-lactamase and MF. (a) Titration of 12 μM Zn(II)–MF in 10 mM Hepes, pH 7.5, with various apoenzyme concentrations (0.24, 1.8, 3.5, 5.3, 7.1, and 8.8 μM), at 25 °C. The corresponding [E]/[Zn(II)–MF] ratios are indicated. (b) Absorbance change at the maximum of the peak (365 nm) corresponding to the metal-free compound [thick line in (a)].

stoichiometry of the two enzymes. With CcrA, Fast *et al.* were unsuccessful in preparing a monozinc enzyme and concluded that the two zinc ions bound cooperatively, which again suggests that the dizinc form is the only catalytically active species. These observations contrast with previous findings (see, e.g., Refs. 27 and 33) that both enzymes are active as the mononuclear and the binuclear forms and use different mechanisms in the two possible states.

The question as to how many zinc ions are required for catalytic activity, and hence what is the physiologically relevant enzyme species that is to be considered as a target, is important since it represents a central issue for the design of potent inhibitors against metallo-β-lactamases. In this context, it is interesting to recall that strong positive cooperativity is observed even for cadmium binding in the presence of the inhibitor thiomandelate.[43] Although the intracellular concentration of free zinc is expected to be very low (below 10^{-12} M in both eukaryotic and bacterial cells[29]), very little information is available concerning extracellular fluids and infection foci, where zinc ion concentration might be significantly higher. For example, total zinc concentrations of 9 mM and 25 μM have been reported in human prostatic and seminal fluid[44] and in human sweat,[45] respectively, but the concentration of free ions has not been evaluated.

Finally, just before submitting this article for publication, we note an additional contribution by Selevsek *et al.* on Zn(II) binding to BcII.[38] These authors report MS data that are very consistent with those presented here (with $K_1/K_2 = 3.7$) and thus indicate positive cooperativity. This contrasts, however, with their far-UV CD spectra and competition experiments with MF, which are apparently different from ours. This is surprising since the enzyme preparation they used was actually prepared in this laboratory, by O.J. On the basis of the analysis of their data, Selevsek *et al.* propose that binding is noncooperative and leads to dimerization of the enzyme.[38] The latter hypothesis is inconsistent with our NMR results (Fig. 5) since no broadening of the signals is observed upon zinc titration, which would be expected if dimerization of the protein occurred. On the contrary, the HSQC spectrum (Fig. 5) of the dizinc enzyme species shows a clear sharpening of the observed signals.

Materials and Methods

Enzyme and chemicals

Unless otherwise specified, chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were all reagent grade.

The recombinant *B. cereus* 569/H/9 β-lactamase (BcII), cloned in a pET9a expression vector (containing the kanamycin resistance gene[33]), was transformed into *Escherichia coli* BL21 (DE3) (Novagen, Madison, WI, USA). Following inoculation with 100 mL of an approximately 12-h preculture, the enzyme was expressed in a 60-L fermentor (BioFlo, New Brunswick), containing 50 L of minimal medium (i.e., 14.6 $g \cdot L^{-1}$ Na_2HPO_4, 5.5 $g \cdot L^{-1}$ KH_2PO_4, 1 $g \cdot L^{-1}$ NaCl, 0.24 $g \cdot L^{-1}$ $MgSO_4$, 0.02 $g \cdot L^{-1}$ $CaCl_2$, 1 $g \cdot L^{-1}$ NH_4Cl, and 10 $g \cdot L^{-1}$ D-glucose) and 50 $\mu g \cdot mL^{-1}$ of kanamycin. The culture was grown at 37 °C, pH ~7, and cell development was followed by monitoring both oxygen consumption and turbidimetry. At $A_{600} \approx 0.6$, expression was induced by addition of 1 mM IPTG, and cell growth was allowed to continue for another 12 h at 37 °C. The cells, harvested by centrifugation (5000*g* for 20 min at 4 °C), were resuspended in 1.2 L of 10 mM Mes, pH 6.0, containing 1 mM $ZnSO_4$ (buffer A), and lysed with the help of a disintegrator (Series Z, Constant System, Warwick, UK). After centrifugation (9000 *g* for 30 min at 4 °C), the supernatant containing the periplasmic proteins was loaded onto a 150-mL SP Sepharose Fast Flow column (GE Healthcare, Uppsala, Sweden), equilibrated in buffer A. BcII was eluted with a linear NaCl gradient (0 to 1 M) in buffer A and the enzymatic activity of the fractions was assayed using benzylpenicillin as substrate. The active fractions were pooled and NaCl was removed by dialysis in buffer A. A second purification step was performed using a UNO 12S column (Biorad, Hercules, CA, USA), equilibrated in buffer A. Elution of the enzyme was achieved as above, and the active fractions were pooled, dialyzed in 10 mM Hepes, pH 7.5, containing 300 mM NaCl and 100 μM $ZnCl_2$, and finally concentrated (2.1 $mg \cdot mL^{-1}$). No trace of contaminating protein was found, either by SDS-PAGE, by N-terminal amino acid sequencing (S-Q-K-V-E), or by MS analysis ($M_r = 24{,}960$ in the absence of zinc ions), indicating a homogeneity higher than 98%. Both N-terminal and MS results indicated that the recombinant enzyme was identical with the original *B. cereus* 569/H/9 β-lactamase. The protein concentration was determined by absorbance measurements at 280 nm, using an extinction coefficient value of 30,500 $M^{-1} \cdot cm^{-1}$.[27] The final preparation was stored at −20 °C in 1-mL aliquots (2 $mg\ mL^{-1}$).

^{15}N-labeled enzyme was expressed and purified as described previously.[46]

Preparation of the apoenzyme

The metal-free BcII β-lactamase was prepared as follows: the enzyme (10 mL, 20 μM) was dialyzed, while stirring, against two changes of 10 mM Hepes (1 L), pH 7.5, in the presence of 300 mM NaCl and 20 mM EDTA over 12-h periods at 4 °C. EDTA was subsequently removed by using two dialysis steps against 10 mM Hepes, pH 7.5, containing 1 M NaCl and finally two dialysis steps against the same buffer containing 300 mM NaCl only. The resulting apoenzyme solution contained less than 1% of metal-bound enzyme, as judged by enzyme activity measurements and ESI–MS experiments (see Results and Discussion). Furthermore, ESI–MS measurements showed no enzyme-bound EDTA, whereas NMR measurements indicated a [free EDTA]/[BcII] ratio less than 0.1.

CD measurements

CD measurements in the far-UV region (190–250 nm) were performed at 25 °C, with a Jasco J-810 spectropolarimeter (Jasco, Japan), using a protein concentration of 0.4 $mg \cdot mL^{-1}$ (16 μM) and a 0. 1-cm cell path length. Unless mentioned otherwise, the buffer used was 10 mM Hepes, pH 7.5, containing 300 mM NaCl and various concentrations of zinc ($ZnSO_4$). Spectra were acquired at a scan

speed of 50 nm·min^{-1}, with a 1-nm bandwidth and a 4-s integration time. The spectra were averaged after 10 accumulations and corrected by subtracting the buffer spectrum obtained under the same conditions.

Deconvolution of the CD spectra was performed using the CDSSTR program and the protein data sets no. 4 and 7, found in DICHROWEB,[47,48] a facility of the Biotechnology and Biological Sciences Research Council Centre for Protein and Membrane Structure and Dynamics.

Kinetic measurements

Hydrolysis of benzylpenicillin (Sigma-Aldrich) and cephalexin (Johnson & Johnson, Langhorne, PA, USA) was monitored by following absorbance changes at 235 and 260 nm, respectively, using a Kontron double-beam Uvikon 940 spectrophotometer equipped with a thermostatically controlled cell holder and a 1. 0-cm cell path length. Values of the kinetic parameters k_{cat} and K_m were determined at 30 °C in 10 mM Hepes, pH 7.5, by measuring initial rates for 12 substrate concentrations and by fitting the data directly to the Henri–Michaelis–Menten equation, using John Easterby's Hyper software†.

In a typical titration experiment, 0.16 mg·mL^{-1} (6.2 μM) of apoBcII was added to 100 μM cephalexin containing various zinc concentrations (0 to 24.8 μM, i.e., 0 to 4 equivalents) in a quartz cuvette. The activity was measured immediately, as described above.

Competition with MF

Competition experiments were performed with the chromophoric chelator MF {2-[2-(5-carboxy)oxazole]-5-hydroxy-6-aminobenzofuran-*N*,*N*,*O*-tri-acetic acid, also known as Furaptra; Molecular Probes, Eugene, OR, USA}, using a Uvikon 940 spectrophotometer. The indicator was initially titrated with Zn(II), by monitoring changes in absorbance at 365 nm (extinction coefficient of Zn-MF at 363 nm = 28,500 M^{-1}·cm^{-1} [33]) until a 1:1 [Zn(II)]/[MF] ratio was obtained. A 143-μM solution (500 μL) of this complex was then back-titrated by stepwise addition of 20 μL apoenzyme, and changes in absorbance were monitored at 365 nm, in 10 mM Hepes pH 7.5, at 25 °C. Dilution of the MF solution upon addition of BcII was taken into account.

Mass spectrometry

MS experiments were performed under non-denaturing conditions, that is, in the absence of acetonitrile. Under these conditions, a narrow distribution of charge states was observed (i.e., states 10+ and 11+ only), confirming that the protein remained folded. Samples were prepared to obtain 10 μM apoBcII in 10 mM ammonium acetate, pH ~7. Buffer exchange and desalting were performed by exhaustive dialysis of the apoenzyme (15–20 μM) against 10 mM ammonium acetate, pH 7.0, using a Slide-A-Lyzer Dialysis Cassette (extra strength, 10,000 molecular weight cutoff, Pierce, Rockford, IL, USA). Different concentrations of Zn(II) ($ZnSO_4$, from 0 to 22.2 μM) and Cd(II) ($CdSO_4$, from 0 to 30 μM) were added. MS experiments were performed on a Q-TOF Ultima Global (Micromass UK Limited, Manchester, UK) electrospray mass spectrometer. The z-spray source was operated in the positive ion mode at a capillary voltage of 2.8 kV. The source block temperature was 100 °C, and the desolvation gas (N_2) temperature was 150 °C. The RF Lens 1 voltage was set to 100 V. All mass spectra were acquired and processed with MassLynx 4.0 (Service Pack 1) from 1000 to 3000 m/z. Interesting peaks were centroided with suitable parameters in order to obtain a vertical line passing through the center of gravity of each isotopic distribution. The centroid top (80%) and the experimental resolution (10,000) were the same for all isotopic distributions, whereas the number of channels was optimized for each isotopic distribution. The resulting centered spectra give the center of the isotopic distributions and their "areas". These latter correspond to the sum of the intensities of the points across the peaks in the continuum trace, and they were used to calculate the relative abundances of each species.

Nuclear magnetic resonance

For NMR, cadmium and zinc titrations were performed at room temperature, by gradually adding microliter volumes (1–10 μL) of 0.05–0.1 M $^{113}CdCl_2$ (95.83% enriched, Cambridge Isotope Ltd.) or $ZnCl_2$ to the β-lactamase apoenzyme (1.0–2.0 mM) in 20 mM Mes and 100 mM NaCl, pH 6.4. The final sample volume was 450 μL. NMR spectra were acquired at 25 °C using Bruker Avance DRX or DMX 600-MHz instruments. Backbone NH resonances were observed by ^1H–^{15}N HSQC with States TPPI and Watergate.[49] The imidazole $^{15}N(C)H$ resonances were observed by ^1H–^{15}N heteronuclear multiple quantum coherence (HMQC) as described previously.[46]

Equilibrium model for zinc binding

Metal binding on the two sites (i.e., histidine and cysteine sites) of the enzyme is described by four microscopic dissociation constants.[27] Our experiments cannot, however, discriminate between binding to the two sites, and thus, only two macroscopic equilibria are considered in this work:

$$E + M \rightleftharpoons EM \qquad K_1 = [E] \cdot [M]/[EM] \tag{2}$$

$$EM + M \rightleftharpoons EM_2 \qquad K_2 = [EM] \cdot [M]/[EM_2] \tag{3}$$

where M stands for the zinc ions, E stands for the enzyme, and K_1 and K_2 are the dissociation constants. For a given set of equilibrium constant values, the equilibrium concentrations of the different enzyme species (i.e., E, EM, and EM_2) can be derived and compared with the experimentally determined values.

Data analysis

The program SigmaPlot version 9 (Systat Software, San Jose, CA, USA) was used for nonlinear least-squares analysis of the data. Errors are calculated as standard deviations throughout.

† http://www.liv.ac.uk/%7Ejse/software.html

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