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ARTICLE

pH and Basicity of Ligands Control the Binding of Metal-ions to *B.cereus* B1 β -Lactamase

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Hasina Motara, Dharmit Mistry, David R. Brown, Robert A. Cryan and Michael I. Page*

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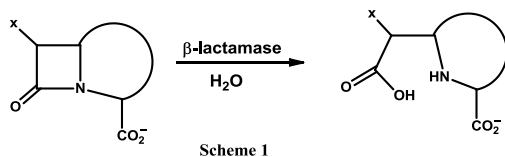
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Metallo- β -lactamases (MBLs) are a group of enzymes responsible for a significant proportion of bacterial resistance to β -lactam antibiotics, by catalysing the hydrolysis of the β -lactam. The MBL from *B. cereus* BcII (569/H/9) forms both mono- and bi-nuclear species with native zinc and also with cobalt and cadmium ions. Which species is formed is controlled by the pH because some of the protein ligands used for metal-ion binding must lose a proton to form the metallo-enzyme from the apo-enzyme. The protonation states of the ligands that are used to bind various metal-ions in BcII have been determined using isothermal titration calorimetry (ITC) at different pH's maintained by different buffers. If a single equilibrium is established, by positive cooperative binding, between two metal-ions and the enzyme to form the bi-nuclear enzyme it requires the solution of a cubic equation to model the ITC data. At pH 5.2 and 5.6 the dominant species for all three metals is the mono-nuclear MBL, but only with the native mono-nuclear ZnBcII is the metal-bound water ionised. Compared with ZnBcII, fewer protons are released upon formation of mono-nuclear CoBcII and CdBcII which probably involves metal ion binding to the DCH site. For all three metal-ions, the number of metal-ions binding per molecule of enzyme increases from one to two as the pH is increased from pH 5.20 to 7.20. Only cadmium shows distinct sequential binding in the ITC outputs and the two cadmium ions bind independently and non-cooperatively, showing very distinct and different binding parameters. The apparent single ITC titration curves when two zinc- or cobalt-ions are bound to the β -lactamase are, in fact, not indicative of strong cooperative binding, but are best modelled by two sequential binding steps. The binuclear Cd₂BcII is formed with the release of only two protons from the apo-enzyme and, at pH 7.2, probably has an unionised water bridging the two metal-ions, whereas in Zn₂BcII and Co₂BcII it is a bridged hydroxide-ion, giving rise to the release of three protons on formation of the binuclear enzyme.

1. Introduction

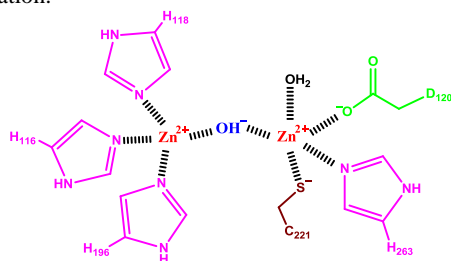
β -Lactam antibiotics are still the most commonly prescribed drugs for the treatment of bacterial infections but many bacteria have developed a number of mechanisms to confer them with antibiotic resistance, the most common and effective of which is the production of β -lactamases^{1,2}. These enzymes catalyse the hydrolysis of the β -lactam thereby inactivating the antibiotic (Scheme 1)³. From a mechanistic point of view, β -lactamases are divided into two classes: serine β -lactamases (classes A, C and D β -lactamases)⁴, which use an active site serine residue for catalysing hydrolysis, and metallo- β -lactamases, (MBLs), (class B β -lactamases), which require one or two zinc ions for catalytic activity⁵. MBLs have been further divided into three subclasses, B1,



B2 and B3, based on their amino acid sequences, substrate profile and metal-ion requirement⁶, so that enzymes from each class exhibit specific functional and mechanistic properties^{4,7}. In particular, while the B1 and B3 enzymes exhibit maximum activity as di-zinc species, the B2 β -lactamases are inhibited upon binding of a second Zn(II) ion⁸. MBLs catalyse the hydrolysis of most classes of β -lactam antibiotics including penicillins, cephalosporins and carbapenems, but there are no clinically useful inhibitors of MBLs⁹, which are also insensitive to the known inhibitors of the serine β -lactamases^{10,11}. Carbapenems are potent broad-spectrum antibiotics that are reserved for life-threatening bacterial infections but their effectiveness is increasingly compromised by resistance particularly due to the carbapenem-hydrolyzing metallo- β -lactamase NDM-1¹².

The majority of metallo- β -lactamases belong to subclass B1 (e.g. BcII, SPM-1, CcrA, BlaB and the IMP and VIM families), but even within this group different enzymes have been reported to vary widely in some details of their catalytic mechanism and in their affinity for zinc^{2,13,14}. The most studied MBL is that from *Bacillus cereus*, BcII, and serves as a model B1 enzyme with a characteristic $\alpha\beta/\alpha$ fold, a metal-binding motif¹⁵ and an active site located at the bottom of a solvent-accessible crevice, bounded by several flexible loops¹⁶ of which L7, L9 and L12 contain the metal-binding ligand residues. There are two binding sites for Zn(II) ions in BcII, referred

to as the 3H (site1) and DCH (site 2) sites, with the former metal-binding residues being His116, His118, and His196 and the latter Asp120, Cys221, and His263 (**Scheme 2**)¹⁷. The metal to protein stoichiometry is apparently uncertain as different findings have been reported, particularly by X-ray crystal structures obtained under various experimental conditions¹⁸. The X-ray crystal structure of the SPM-1 β -lactamase has been determined with only a single Zn(II) ion in the 3H site¹⁸. By contrast, the crystal structures of CcrA¹⁹, IMP-1²⁰ and BlaB²¹ β -lactamases show binuclear enzymes with zinc ions occupying both sites²². In the latter zinc1 is in the 3H site and has a distorted tetrahedral geometry, bound to three His residues and a bridging water molecule/hydroxide ion at a variable distance from Zn2 in different structures¹⁸. The second Zn in the DCH site has distorted trigonal-bipyramidal geometry, with three equatorial ligands (Cys221, His263 and the bridging water) and Asp120 and a second water molecule in the two axial positions (**Scheme 2**)²³. Structures of the BcII^{15,22,24} and VIM-2²⁵ enzymes show either only the histidine site or both sites occupied, dependent on the conditions used for isolation.



Scheme 2 Schematic diagram of the active site structure of binuclear Zn β -lactamase BcII¹⁶

Despite the knowledge of the crystal structures, data from numerous spectroscopic techniques and extensive kinetic studies, the mechanism of the MBL catalysed hydrolysis of β -lactams remains controversial, particularly concerning the role and necessity of each metal ion. The mononuclear form of SPM-1 is catalytically active, but both mono- and bi-nuclear BcII have been reported to be active^{26,27}. A major problem *in-vivo* is knowing the concentration of zinc available for binding as there is considerable disagreement among the measurements of accessible Zn^{2+} in cells using different tools, with estimates varying by 3 orders of magnitude and ranging from 5 to 10^3 pM^{28,29,30}. The precise concentration of accessible Zn^{2+} , its spatial distribution, and the conditions under which levels are dynamic are poorly understood³¹. Not only does the actual metal to protein stoichiometry of MBLs remain controversial³² but large variations in zinc affinity among the B1 enzymes *in vitro* have been reported together with a wide range of values for the sequential binding constants for BcII (**Scheme 3**, E= enzyme, M= metal-ion) ($K_1 \sim 10^4 M^{-1}$ to $10^9 M^{-1}$ and $K_2 \sim 10^2 M^{-1}$ to $10^6 M^{-1}$)^{26,33,34}. If the binding constant $K_1 > K_2$, i.e. the second zinc-ion binds more weakly than the first then both the mono- and bi-nuclear species will be detectable. If the converse is

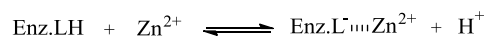


Scheme 3

true, i.e. $K_2 \gg K_1$, then only the binuclear enzyme will be formed, consistent with positive cooperativity³⁵ and the mono nuclear species may not even be detectable. The apparent variation in these equilibrium parameters and those reported by X-ray crystallography

are probably due to different experimental conditions used such as pH, ionic strength and enzyme concentration. A recent extensive investigation of the binding of zinc to BcII (569/H/9), at pH 6.4 (NMR) and pH 7 (MS), showed that two zinc ions bind cooperatively and that the di-zinc enzyme is the only one relevant to catalysis³⁶.

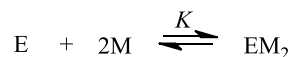
One of the obvious variables in these studies is the pH and we have previously shown how BcII activity decreases dramatically with pH³⁷ which can be partially overcome by increasing concentrations of metal-ion^{38,39,40}. For any ionisable amino acid side chain acting as a ligand L⁻ for metal-ion coordination and for which the pK_a of LH is above the pH of the solution, there is potential competition between metal-ion binding and protonation (**Scheme 4**). If the pK_a of LH is greater than the pH and its conjugate base L⁻ is a good Lewis base for metal-ion coordination, it is likely to suffer competition from protonation. Conversely if the pK_a of LH is low



Scheme 4

there is less rivalry with protonation but L⁻ will be a weaker binder to the metal-ion. Therefore there is an evolutionary pressure to find ligands of suitable pK_a that balance these opposing effects, determined by the pH range over which the metallo-enzyme is required to be active.

In this work, we are interested in determining the protonation states of the ligands that are used to bind various metal-ions in the metallo- β -lactamase BcII (569/H/9) using isothermal titration calorimetry (ITC) at various pH's maintained by different buffers. As there are six basic amino acid side chain residues bound to the two zinc-ions and a bridging water, believed to be hydroxide-ion, there is a potential for at least one and up to seven or more protons to be released from the apo-enzyme upon binding two metal-ions depending on the state of ionisation of these ligands in the apo-enzyme at a particular pH. A related problem reported here is the software used to calculate the thermodynamic parameters for cooperative binding to form a bi-nuclear enzyme with no detectable formation of the mono-nuclear species. Conventional commercial software designed to model the equilibria established in ITC experiments can be used with stoichiometries of one to one, two to one etc. However, in the two to one case, where there are two equilibria between substrate and ligands, the software is frequently written to assume the two equilibria are established independently, so that the reaction of the second ligand with the substrate is unaffected by the reaction of the first. In this work we have used our own software to model sequential reactions of two ligands with a substrate, as in **Scheme 3**. In addition the hypothetical case of positive cooperativity has been modelled where there is a single equilibrium established between two metal-ions and an enzyme (**Scheme 5**), the solution to which involves a cubic equation. This is then used to analyse the ITC data to distinguish between sequential and 'concerted' formation of the binuclear enzyme. The two models are also compared when the two successive equilibria have variable ratios of binding constants K_2/K_1 .



Scheme 5

2. Results and Discussion

2.1 Enzyme preparation and activity

The recombinant *B. cereus* 569/H/9 β -lactamase (BcII), which was cloned in a pET9a expression vector containing the kanamycin

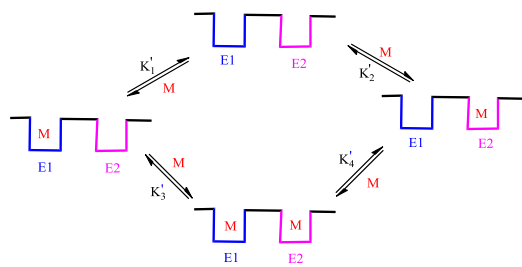
resistance gene, was transformed into *Escherichia coli* BL21 (DE3) and the enzyme then expressed, isolated and purified as previously described³⁹. The apo BcII β -lactamase was prepared by removing the metal-ion with EDTA over 12-h periods at 4 °C and shown to be metal-free by enzyme activity measurements (< 1%) and ESI-MS ($m/z = 24,960\text{Da}$)³⁴. Using atomic absorption spectroscopy the residual zinc-ion concentrations were < 0.1% in the apo-enzyme and < 0.01ppm in the buffers. There was no enzyme-bound EDTA as shown by ESI-MS and less than 10% free EDTA as shown by ¹H NMR⁴¹.

Catalytic activities for the zinc, cadmium and cobalt enzymes were determined using the hydrolysis of benzylpenicillin or cephalixin as substrates from pH 5-8 as previously described⁴¹. For example, for zinc BcII using benzylpenicillin as the substrate at pH 7.0 and 30°C $k_{\text{cat}}/K_m = 8.7 \times 10^5 \text{ M}^{-1}\cdot\text{s}^{-1}$, indicative of full activity of the enzyme preparation.

2.2 Binding various metal-ions to apo metallo- β -lactamase BcII

We are interested in using Isothermal Titration Calorimetry (ITC) to measure the thermodynamic parameters for metal-ion binding to BcII, to investigate the degree of cooperativity in forming the binuclear enzyme with different metal-ions and to identify the state of ionisation of the active-site ligands that bind the metal-ion. The titration involves adding aliquots of metal-ion to a buffered solution of the apo-enzyme in the ITC cell at constant pH and temperature. The ITC technique provides the thermodynamic parameters from a single experiment: the enthalpy change (ΔH°), the reaction stoichiometry (N), the binding constant (K_b) and hence, indirectly, the entropy change (ΔS°). The main principle of ITC is to monitor the enthalpy change associated with a chemical event brought about by titrating one solution into another and the key is to identify the source of the heat absorbed or released. The ITC output is in the form of a plot of power against aliquot of titrant added which is then integrated and the heat (q) values are then re-plotted against the molar ratio of added titrant metal ion to the enzyme in the cell. The stoichiometry of the reaction is determined from the position of the equivalence point whereas the binding constant K_b controls the shape of the curve - the greater K_b the steeper the plot.

When there are two metal-ion binding sites, as in BcII, formation of the mono-nuclear enzyme EM and the binuclear species EM₂ can potentially involve four species with their relevant microscopic binding constants (**Scheme 6**). Binding of the first metal-ion could occur exclusively at site E1 or E2 or be distributed between the two sites; if there is preference for one site, say E1, then the microscopic binding constant $K'_1 > K'_2$. If there is substantial cooperative binding such that the mono-nuclear enzyme is not significantly populated and effectively only the binuclear enzyme EM₂ is formed, then $K'_3 > K'_1$ and therefore $K'_3 > K'_2$ i.e. the binding of the second metal-ion to



Scheme 6

site E2 to give the binuclear enzyme is more favourable when site E1 is already occupied by the first metal-ion compared with binding to the free enzyme to form the mono-nuclear enzyme at the same site E2.

The observed position of the macroscopic equilibrium (**Scheme 3**) is determined by a combination of these microscopic equilibrium constants (K' **Scheme 6**) and the concentrations of metal-ions and enzyme. The usual way of interpreting ITC plots is to model them, and to optimise the fit of the model in terms of the stoichiometry of the reaction, the binding constants and the molar enthalpies of reaction. If the observed binding constants are different with, say, $K_1 > K_2$ (**Scheme 3**) and there are different enthalpies of binding the metal-ions, then two separate events will clearly be seen in the ITC output. The binding constants and molar enthalpies of reaction can be elucidated from a model based on two simple quadratic equations to determine the concentrations of EM and EM₂ throughout the titration. If strong cooperative binding occurs, i.e. macroscopic binding constant $K_2 \gg K_1$ (**Scheme 3**), the equilibrium concentration of EM will be close to zero throughout as the enzyme will effectively be converted directly to EM₂. Therefore an alternative approach to modelling cooperative binding is to consider the single equilibrium (**Scheme 5**) and the associated binding constant K given by **eqn.1** where E, M and EM₂ are the equilibrium concentrations. In this case, the concentration of EM₂ can be calculated by solving the cubic equation (**eqn 2**), where E₀ is the initial total concentration of enzyme and M₀ the total concentration of metal-ion added at any time during the titration and the ITC output calculated from this. This equation has three roots for [EM₂], but the required root can be identified as the (only) root that lies between zero and [M]₀/2 (details given in Electronic Supplementary Information (ESI)).

$$K = \frac{[\text{EM}_2]}{[\text{E}][\text{M}]^2} \quad (1)$$

$$K = \frac{[\text{EM}_2]}{([\text{E}_0] - [\text{EM}_2])([\text{M}_0] - 2[\text{EM}_2])^2} \quad (2)$$

For every injection made in the titration, heat is either absorbed or released and is proportional to the change in concentration of bound metal ion, **eqn.4**, where V is the volume of reaction and ΔH_{obs} is the observed enthalpy of binding. The cumulative heat Q is then

$$q = V \cdot \Delta H_{\text{obs}} \cdot \Delta[\text{M}]_{\text{bound}} \quad (4)$$

expressed as the differential heat output on addition of metal ion and is used to perform the simulation of the titration curve. The shape of the titration curve and the sharpness of the inflection at the equivalence point are dependent on the Wiseman c-parameter⁴² and values of between 10 and 50 were used where possible.

The two models - sequential binding (**Scheme 3**) and the single equilibrium formation of EM₂ (**Scheme 5**) - were used to calculate the fraction of enzyme bound as EM and EM₂ formed as a function of the molar ratio M:E using different ratios of K_2/K_1 and enthalpies of binding to simulate the ITC titration curves (Figures 1-15, ESI). Of particular interest are values of $K_2/K_1 = 0.1 - 10$ where analytical methods may be inadequate to distinguish between the two models. For example, when $K_2/K_1 = 5.0$ more enzyme is converted to combined concentrations of EM and EM₂ in the sequential model using $K_1 = 1 \times 10^6 \text{ M}^{-1}$ and $K_2 = 5 \times 10^6 \text{ M}^{-1}$ than in the concerted model with $K = 5 \times 10^{12} \text{ M}^{-2}$ at a given molar ratio M:E. The differential heats per injectant against the molar ratio M:E for the two models can also be predicted using different enthalpies of binding. When $K_2/K_1 < 10$ there is a distinct difference in the predicted ITC outputs for the two models and consequently they can be used to differentiate them by modelling the experimental data.

In order to show that the ITC output reflected just reactions involving the binding process including any heat formed by protons released being neutralised by the buffer, various control experiments were conducted. Blank titrations were performed in which solutions of each of the three metal ions zinc, cadmium and cobalt were titrated into solutions of each of the buffers used, and no measurable heat was detected in any of the cases, other than that due to dilution of the metal ion. In addition, no heat was detected due to interactions between the enzyme and each of the buffers in similar control titrations in which buffer solutions were titrated into enzyme solution.

2.2(i) Titration of cadmium ion against apoBcII.

Cadmium BcII is an active β -lactamase enzyme which shows a typical bell-shaped rate-pH profile with a maximum value of k_{cat}/K_m around pH = 8.8⁴¹. The maximum value of k_{cat}/K_m for the hydrolysis of cephalixin by Cd BcII is about 10-fold greater than that catalysed by the zinc enzyme at pH=7.5-8.5 indicating that the measured rates represent the activity of the Cd enzyme. A combination of NMR and PAC spectroscopies has shown that Cd BcII binding is a non-cooperative process, with successive population of the mono and binuclear cadmium enzyme.⁴³ Cd BcII is therefore a good species for ITC to identify the two separate binding steps and the titration isotherms at pH 6.80 and 7.20 (Figure 1) show two distinct binding events. This means two cadmium ions bind sequentially to apoBcII

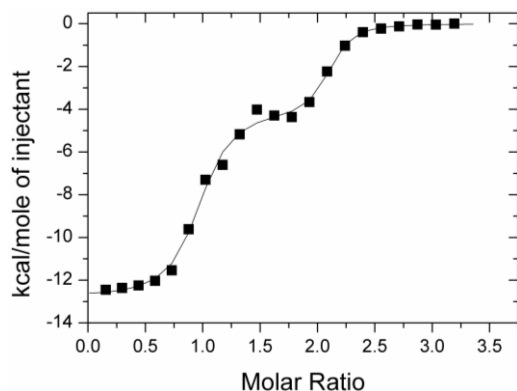


Figure 1 ITC output for CdCl_2 solution (3.6×10^{-4} M) titrated into apoBcII solution (1.8×10^{-5} M) in HEPES buffer (20mM) at pH 7.20 $I=0.1\text{M}$ (KCl) at 25°C.

in a non-cooperative way, with two distinct steps, each of which corresponds to one cadmium ion per mole of enzyme. The stoichiometric ratio for the first binding site at pH 7.20 is 1.02 (± 0.2):1 (Cd^{2+} : Enz) with a binding constant $K_1 = 3.68 (\pm 0.5) \times 10^8 \text{ M}^{-1}$ and for the second binding site the ratio is 0.9 (± 0.2):1 (Cd^{2+} : Enz) with a $K_2 = 6.18 (\pm 0.5) \times 10^6 \text{ M}^{-1}$, which thus shows that BcII has a 70-fold lower affinity for binding the second cadmium compared with the first metal-ion binding. At pH 5.20 in MES buffer the calorimetric output for cadmium ion titration into apoBcII shows a *single* binding endothermic isotherm with a stoichiometric ratio of 0.9 (± 0.2):1 (Cd^{2+} : Enz), $K_1 = 1.28 (\pm 0.6) \times 10^6 \text{ M}^{-1}$ (Table 1).

The number of cadmium ions binding per molecule of enzyme increases from one to two as the pH is increased from 5.20 to 7.20 and the two cadmium ions bind independently and non-cooperatively, showing very distinct and different binding parameters. When one cadmium ion binds to BcII at lower pH, the binding is weaker by nearly three orders of magnitude compared with that at higher pH for the binding of the first metal-ion. Even the second Cd is bound to BcII about 10-fold better at pH 7.2 compared with the single metal bound at pH 5.2. The binding constants are

independent of the nature of the buffer at the same pH and so this difference in binding constants for the formation of the mono-nuclear Cd enzyme suggests different equilibria are involved at pH 7.2 and pH 5.2. If binding was controlled by the ionisation of just one ligand (Scheme 4) then the maximum difference in binding constants between pH 5.2 and 7.2 would be two orders of magnitude. The observed difference of three orders of magnitude indicates that the ionisation of more than one group is required for metal-ion binding at the lower pH. It is also compatible with, at higher pH, the first Cd binding at the DCH site or rapidly exchanging between the two sites as previously suggested⁴³, but at the 3H site at lower pH, possibly due to the lack of ionisation of the Cys221 (Cys-His pair) at lower pH which decreases the binding at DCH site compared with the 3H site.

The observed molar enthalpies of binding vary at a fixed pH maintained by different buffers (Table 3 ESI) which is also indicative that protons are released upon cadmium binding. The heat change generated by the neutralisation by the buffer of the released protons contribute to the observed total enthalpy changes which thus differ depending on the enthalpies of ionisation of the various buffers. These results are discussed later and the data used to elucidate the state of ionisation of the ligands binding the active-site metal-ions.

2.2(ii) Titration of zinc ion against apoBcII.

Aliquots of zinc-ion were titrated against buffered solutions of the apo-enzyme BcII in the ITC cell at constant pH and 25°C (Table 1 ESI). At pH 5.2 the calorimetric output for zinc ion titration into apoBcII shows just a single binding isotherm with a stoichiometric ratio of 1.1(± 0.2):1 (Zn^{2+} : Enz), $K_1 = 1.24 (\pm 0.5) \times 10^6 \text{ M}^{-1}$ (Tables 1 and 2) indicative of formation of predominantly the mono-nuclear enzyme EM. As the pH is increased from pH 5.20 to 7.20, the ITC titrations show, as with cadmium, that the number of zinc-ions binding per molecule of enzyme increases from one to two (Table 2). However, they all show apparent *single* titration curves indicative of a single binding event even with a stoichiometric ratio M:E of two (Figure 2), initially thought to be consistent with the formation of the bi-nuclear enzyme EM_2 with no detectable formation of the mono-nuclear species EM, in agreement with recent studies^{36,38}. However, it is not clear whether the ITC data can be analysed using

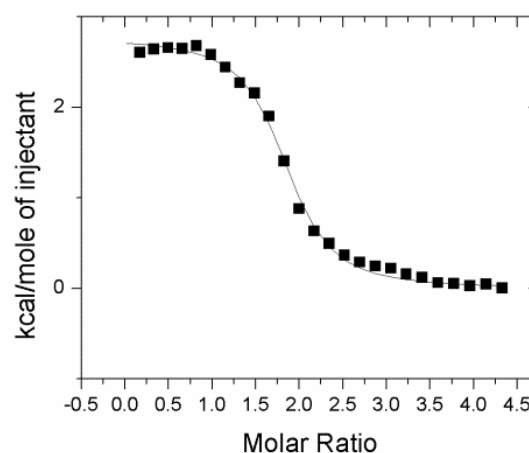


Figure 2 ITC output for ZnSO_4 solution (3.6×10^{-4} M) titrated into apoBcII solution (1.8×10^{-5} M) in MES buffer at pH 6.35 at 25°C.

conventional quadratic equations to elucidate the binding constants or if a cubic approach is necessary by using the simple equilibrium (Scheme 5) in which the bi-nuclear species EM_2 is formed with no

detectable intermediacy of the mono-nuclear EM. We have used our own software to model sequential reactions of two ligands with a substrate (**Scheme 3**). The case where there is a single equilibrium established between two metal-ions and the apo-enzyme (**Scheme 5**) was also modelled which involves the solution of a cubic equation (ESI). For this case, the ITC trace is best simulated in terms of a single dissociation constant for the 'concerted' formation of EM₂ in the 2:1 reaction.

Modelling the data for apparent formation of only the binuclear enzyme (**Scheme 6**) using the cubic equation for positive cooperative binding, which assumes there is no mono-nuclear species formed at higher pH, does *not* give a good fit (**Figure 3**). This indicates that there is sequential binding with both mono- and binuclear species formed but that the observation of just one apparent binding event implies either the two binding sites have similar dissociation constants, or that binding of zinc to the first, tighter site, gives the same heat response as the binding of the second weaker site. The binding constants at pH 7.20, for the formation of EM and EM₂ are $K_1 = 1.69 (\pm 0.4) \times 10^8 \text{ M}^{-1}$ and $K_2 = 5.24 (\pm 0.4) \times 10^6 \text{ M}^{-1}$, respectively (**Table 1**). From this ITC data there is not substantial positive cooperative binding of zinc ions to BcII and there is a significant proportion of the mono-nuclear species EM present, in contrast to a recent report³⁷.

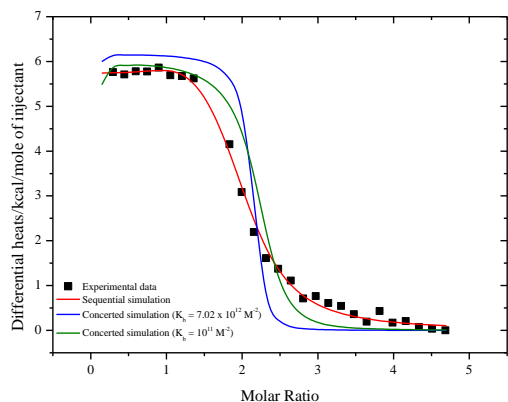


Figure 3 Isotherm of ZnSO₄ solution ($3.6 \times 10^{-4} \text{ M}$) titrated into apo-BcII solution ($1.7 \times 10^{-5} \text{ M}$) in cacodylate buffer at pH 6.35 at 25°C, experimental data (■) and best fit parameters for sequential model (—) $K_1 = 1.37 \times 10^7 \text{ M}^{-1}$, $\Delta H_1 = 5764 \text{ cal mol}^{-1}$, $K_2 = 5.14 \times 10^5 \text{ M}^{-1}$ and $\Delta H_2 = 6746 \text{ cal mol}^{-1}$ and 'concerted' model (—) $K = 7.02 \times 10^{12} \text{ M}^{-2}$ (—) $K = 1.0 \times 10^{11} \text{ M}^{-2}$, $\Delta H = 12.51 \text{ kcal mol}^{-1}$.

2.2(iii) Titration of cobalt ion against apoBcII.

The kinetics of the hydrolysis of benzylpenicillin catalysed by cobalt substituted BcII β -lactamase are biphasic and the dependence of enzyme activity on pH and metal-ion concentration indicates that only the di-cobalt enzyme is catalytically active⁴⁰. A mono-cobalt enzyme species is formed during the catalytic cycle, which is virtually inactive and requires the association of another cobalt ion for turnover. Using the hydrolysis of cephalixin as a substrate, cobalt BcII shows a bell-shaped pH-rate profile and remarkably is about 100 fold more active than the native ZnBcII at pH 7.0⁴¹. For imipenem as a substrate, it appears that there is some catalysed hydrolysis by the mono-nuclear Co-enzyme at pH 7.5⁴⁴.

The ITC titrations of cobalt-ions against apoBcII show (Table 3 ESI), as with both zinc and cadmium, that the number of metal-ions binding per molecule of enzyme increases from one to two as the pH is increased from pH 5.20 to 7.20 (**Table 2**). At pH 5.20, the binding constant for formation of the mono-nuclear cobalt BcII $K_1 = 1.68$

(± 0.3) $\times 10^5 \text{ M}^{-1}$. Similar to zinc, the cobalt-ion titrations show apparent single titration curves even when two metal-ions are bound to the enzyme (**Figure 4**). However, if it is assumed that there is no mono-nuclear species formed at higher pH and only EM₂ is produced (**Scheme 5**), then application of the cubic modelling does not give a good fit. This again probably indicates that there is sequential binding with both mono- and bi-nuclear species formed but that the observation of just one apparent binding event in the ITC titration implies that either the two binding sites have similar binding constants, or that binding of cobalt to the first, tighter site, gives the same heat response as the binding of the second weaker site. At pH 7.20, the binding constants for formation of EM and EM₂ are $K_1 = 4.85(\pm 0.3) \times 10^6 \text{ M}^{-1}$ and $K_2 = 2.36(\pm 0.3) \times 10^6 \text{ M}^{-1}$, respectively (**Table 1**). Again, it does appear that there is not substantial positive cooperative binding of cobalt ions to BcII. Some studies⁴⁵ have suggested the binding of a third cobalt(II) ion at a weaker site at pH 7.5 but there is no ITC evidence to support this.

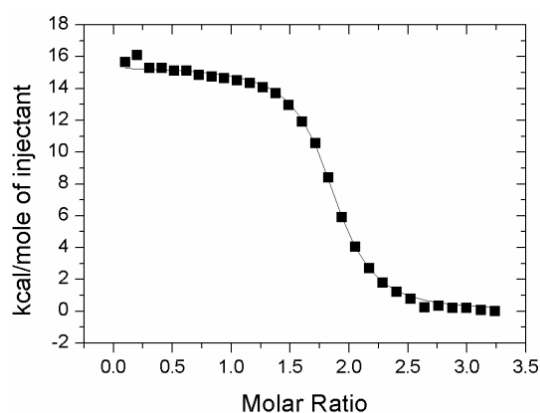


Figure 4 ITC output for CoCl₂ solution ($3.6 \times 10^{-4} \text{ M}$) titrated into apoBcII solution ($1.8 \times 10^{-5} \text{ M}$) in cacodylate buffer at pH 6.80 at 25°C.

The conversion of bi-nuclear Co₂-BcII to the mono-nuclear enzyme as the pH decreases is also manifest in the first-order dependence of the second-order rate constants, k_{cat}/K_m , on cobalt-ion concentration for the Co-BcII catalysed hydrolysis of cefoxitin at low pH, whereas, at higher pH, the rates of hydrolysis are invariant with metal-ion concentration^{40,41}. Both kinetic and ITC observations indicate that protonation of an enzyme ligand that binds cobalt-ion occurs at lower pH and this is responsible for the loss of one cobalt ion from the enzyme active site.

2.3 Dependence of number of metal-ions bound to BcII and their binding constants on pH

The number of metal-ions binding to apoBcII increases from one to two for zinc, cobalt and cadmium as the pH is increased from 5.20 to 7.20. For all three the ratio of metal-ion to apoenzyme (N) varies in a sigmoidal manner with pH (**Figure 5**) indicative of an apparent ionisation process controlling metal-ion binding by an acidic group of $\text{p}K_a$ 6.10, i.e. a protein residue of about $\text{p}K_a$ 6.1 is required in its deprotonated form for binding the second metal-ion. This is in reasonable agreement with the value of 6.5 found from kinetic studies of cobalt BcII catalysed reactions⁴¹. This $\text{p}K_a$ is unlikely to correspond to ionisation of the metal-ion bound water which forms the bridge between the two metal-ions, as it would be expected to vary with the metal-ion^{5, 41}. The pH dependence of the rate constant for the inactivation of apo BcII by iodoacetamide indicates that in

the apo-enzyme the pK_a of the Cys221 (or the Cys221-His263 ion-pair) is 7.85⁴¹. In the presence of a di-positively charged metal-ion in the mono-nuclear species this pK_a will presumably be lower.

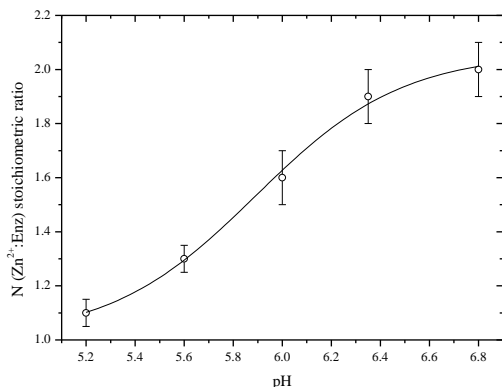


Figure 5 Plot of N (ratio Zn^{2+} :Enz) against pH for zinc ion binding to apoBcII at 25°C.

It is likely, therefore, that the ligand of $pK_a \sim 6.1$ controlling formation of the binuclear species with pH is the thiol of Cys221. Interestingly, some zinc and iron-dependent enzymes that share the metallo- β -lactamase fold but lack cysteine as a metal-ion binding ligand show binuclear zinc-ion binding with tighter binding of the first compared with the second zinc-ion⁴⁶.

A summary of the binding constants for mono- and bi-nuclear metallo- β -lactamases BcII as a function of pH at 25°C obtained by ITC is given in **Table 1**.

Table 1 Summary of the binding constants for mono- and bi-nuclear metallo- β -lactamases BcII as a function of pH at 25°C

pH	Zn^{2+}		Co^{2+}		Cd^{2+}	
	K_1 (M^{-1})	K_2 (M^{-1})	K_1 (M^{-1})	K_2 (M^{-1})	K_1 (M^{-1})	K_2 (M^{-1})
7.20	$1.69(\pm 0.4) \times 10^6$	$5.24(\pm 0.4) \times 10^6$	$4.85(\pm 0.3) \times 10^6$	$2.36(\pm 0.3) \times 10^6$	$3.68(\pm 0.5) \times 10^6$	$6.18(\pm 0.5) \times 10^6$
6.80	$2.04(\pm 0.4) \times 10^6$	$7.12(\pm 0.4) \times 10^6$	$8.30(\pm 0.6) \times 10^6$	$1.80(\pm 0.3) \times 10^6$	$3.53(\pm 0.3) \times 10^6$	$4.08(\pm 0.4) \times 10^6$
6.35	$1.37(\pm 0.3) \times 10^7$	$5.14(\pm 0.3) \times 10^7$	$2.17(\pm 0.3) \times 10^6$	$6.81(\pm 0.5) \times 10^5$	$2.72(\pm 0.3) \times 10^6$	$4.70(\pm 0.3) \times 10^6$
6.00	$9.28(\pm 0.6) \times 10^7$		$1.47(\pm 0.4) \times 10^6$		$3.33(\pm 0.5) \times 10^6$	
5.60	$1.91(\pm 0.4) \times 10^6$		$2.35(\pm 0.5) \times 10^5$		$1.86(\pm 0.5) \times 10^6$	
5.20	$1.24(\pm 0.5) \times 10^6$		$1.68(\pm 0.6) \times 10^5$		$1.28(\pm 0.3) \times 10^6$	

2.4 Enthalpy changes and number of protons released upon metal-ion binding to apoBcII

The observed molar enthalpy change (ΔH_{obs}°) obtained from the ITC titration at constant pH includes heats from several events as the metal-ions bind to the apo-enzyme. These heats include those directly controlling metal-ion binding (ΔH_{bind}°) such as the desolvation of the metal-ion, metal-ion enzyme ligand interactions, the ionisation of any amino acid side chains acting directly as ligands (**Scheme 2**) and others whose pK_a is changed by the new environment, and any conformational changes that the enzyme might undergo when the metal binds. It also includes indirect events such as the neutralisation of the one or more (n) protons released by metal-ion binding to the enzyme and neutralised by the buffer ($\Delta H_{ion(buffer)}^{\circ}$) (**eqn 5**).

$$\Delta H_{obs}^{\circ} = \Delta H_{bind}^{\circ} + n \Delta H_{ion(buffer)}^{\circ} \quad (5)$$

As there are six basic amino acid side chain residues bound to the two zinc-ions and a bridging water, believed to be a hydroxide-ion, (**Scheme 2**) there is a potential for, at least one, $n=1$, and up to seven, $n=7$, or more protons to be released from the apo-enzyme upon binding two metal-ions. The value of n depends on the state of ionisation of these ligands in the apo-enzyme determined by their pK_a and the pH of the solution. When ITC measurements have been made in more than one buffer at constant pH a plot of ΔH_{obs}° against $\Delta H_{ion(buffer)}^{\circ}$ should be linear and give values of n and ΔH_{bind}° . For example, **Figure 6** shows the plot of ΔH_{obs}° against $\Delta H_{ion(buffer)}^{\circ}$ ⁴⁷ for zinc-ions titrated against apoBcII in five buffers at pH 6.80 from which the slope gives $n=2.9 (\pm 0.2)$. The slope is negative because the net result from metal-ion binding is proton release and neutralisation by the basic form of the buffer is the reverse of the ionisation process given by $\Delta H_{ion(buffer)}^{\circ}$.

ITC titrations for each of the three metal-ions were performed at six pH's from pH 5.20 to 7.20 using at least three different buffers at each pH (ESI Tables 1-3). The observed molar enthalpy changes (ΔH_{obs}°) varied with the buffer type and hence ($\Delta H_{ion(buffer)}^{\circ}$). It is clear that metal-ion binding is associated with the release of protons at all pH's studied, whether the mono- or bi-nuclear enzyme is

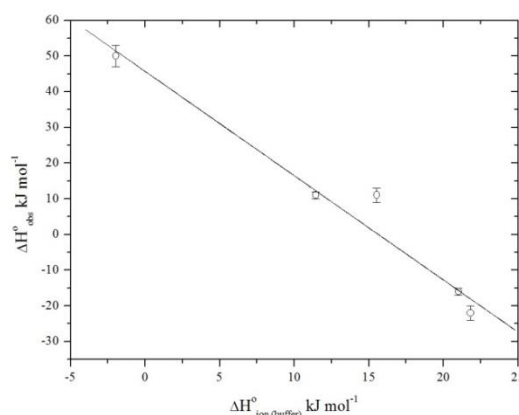


Figure 6 Plot of ΔH_{obs}° against $\Delta H_{ion(buffer)}^{\circ}$ ⁴⁷ at pH 6.80 for zinc-ions titrated into apoBcII solution.

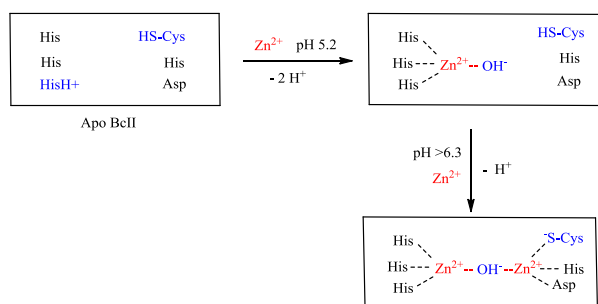
formed. This implies that some of the enzyme ligands required for metal ion binding exist in their protonated state in the apo-enzyme and so metal ion binding to the apo-enzyme represents a competition between the metal ion and the proton for the ligand (**Scheme 4**) and explains the variation of the number of metal ions bound with pH. Consequently there is not a simple stoichiometry N (M^{2+} :Enz) because there is a mixture of mono- and bi-nuclear species present which varies with pH (**Figure 5**). The values for n (number of protons released) at different pHs are subject to considerable error, nonetheless, it is convenient to discuss the results using whole numbers of protons released. Over the pH range 5.2 -7.2, it appears that the maximum number of protons released on forming the binuclear enzyme is three for the native zinc and cobalt enzymes, but probably only two for cadmium BcII (**Table 2**). Assuming that in the binuclear enzyme the bridged water between the two zinc or cobalt ions is in the form of a hydroxide-ion, then one of these protons must be from the metal-bound water leaving two ligands in the protein which requires ionisation for two metal ions to bind. It is possible for the number of protons released to decrease with increasing pH as a ligand in the apo-enzyme may become ionised as the pH exceeds its pK_a . It also seems reasonable that the number of protons released may increase as the number of bound metal ions increases from one to two. All of these changes are in fact reflected

in the variation of n , the number of protons released, with pH (Table 2) and are consistent with them coming from one of the imidazolium ions of one of the histidines in site 3H and the thiol of Cys221 in the DCH site and the bridging water between the two metal-ions.

Table 2 Number of metal ions binding and protons released for zinc, cobalt and cadmium ion binding to apoBcII as a function of pH

pH	Zinc ion		Cobalt ion		Cadmium ion			
	N (Zn ²⁺ :Enz)	n (no of protons released)	N (Co ²⁺ :Enz)	n (no of protons released)	N (Cd ²⁺ :Enz)		n (no of protons released)	
5.20	1.1 (±0.2)	2.1 (±0.2)	0.9 (±0.2)	1.1 (±0.2)	1.0 (±0.2)	-	0.9 (±0.2)	-
5.60	1.3 (±0.2)	1.6 (±0.2)	1.1 (±0.2)	1.4 (±0.2)	1.0 (±0.2)	-	0.8 (±0.2)	-
6.0	1.6 (±0.2)	1.9 (±0.2)	1.3 (±0.2)	1.7 (±0.2)	1.0 (±0.2)	-	2.0 (±0.3)	-
6.35	1.9 (±0.3)	2.0 (±0.3)	1.7 (±0.2)	3.0 (±0.3)	1.0 (±0.2)	1.0 (±0.2)	1.7 (±0.2)	0.2 (±0.2)
6.80	2.0 (±0.2)	2.9 (±0.2)	2.0 (±0.2)	3.1 (±0.3)	1.0 (±0.2)	1.0 (±0.2)	1.8 (±0.2)	0.3 (±0.2)
7.20	1.8 (±0.2)	2.6 (±0.3)	1.9 (±0.2)	2.7 (±0.3)	1.0 (±0.2)	1.0 (±0.2)	1.1 (±0.2)	0.3 (±0.2)

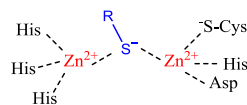
At pH 5.20 the dominant form of the native zinc BcII is the mono zinc enzyme and metal-ion binding to apoBcII releases two protons. This is consistent with one of the histidine residues of the 3H site existing in its protonated form in the apo-enzyme, which upon metal-ion binding to this site releases one proton. Coordination of zinc-ion to the protein lowers the pK_a of the zinc-bound water so that it ionises at pH 5.2 releasing the second proton (Scheme 7)^{5, 37}.



Scheme 7

Above pH 6.3 the dominant species is the binuclear enzyme formed by ionisation of the thiol of Cys221, releasing another proton, and allowing binding of a second zinc to the DCH site.

Cysteiny peptides, including captopril, are reversible competitive inhibitors of MBLs⁴⁸ and an X-ray structure of the complex shows the thiol anion has displaced the bridging hydroxide-ion binding to both zinc-ions (Scheme 8)²¹. The inhibition constants

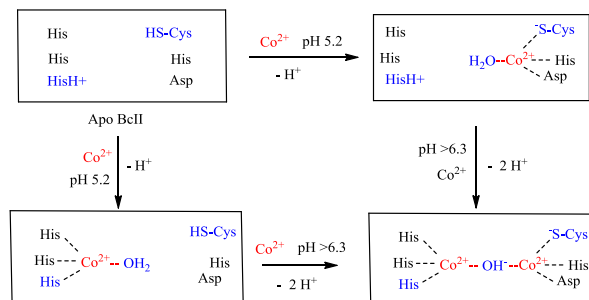


Scheme 8

K_i are pH independent between pH 6 and 9, but increase and become pH dependent at lower pH due to protonation of a group with a pK_a of 5.7 (±0.4)⁴⁸, in very good agreement with the present studies. Protonation of the Cys221thiolate anion decreases the number of zinc-ions bound to BcII decreases from 2 to 1 and so inhibition by binding of the thiol peptides also decreases.

When cobalt ion binds to apoBcII, the number of protons released increases from one to three as the pH is increased. At pH

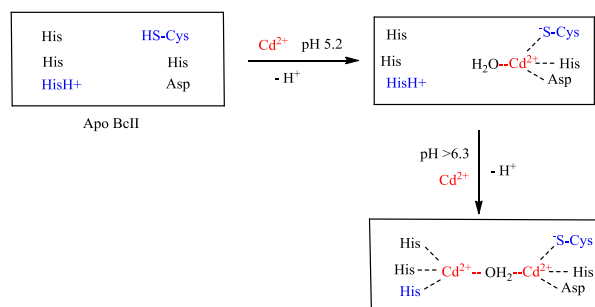
5.2 only one cobalt ion binds and only one proton is released, consistent with binding to either the DCH or 3H site⁴⁵ but with the



Scheme 9

cobalt bound water still unionised (Scheme 9). As the pH is increased two cobalt ions are bound with three protons released in total.

Cadmium ion binding to apoBcII shows distinct mono- and binuclear species. At pH 5.20 when the first cadmium ion binds, only one proton is released, consistent with binding to the DCH site accompanied by ionisation of the Cys 221 thiol. Although the number of cadmium ions bound increases from one to two as the pH is increased, only one more proton is released upon formation of mono-nuclear CdBcII and apparently no more when the second cadmium ion binds to apoBcII. Unlike the three protons released upon formation of the binuclear zinc and cobalt enzymes, with cadmium it appears that only two protons are released in total. This suggests that at pH 7.2 the water molecule bridging the two cadmiums is unionised (Scheme 10), consistent with the pK_{a1} of 8.7 seen in the pH-rate profiles for hydrolysis of β-lactams catalysed by CdBcII⁴¹.



Scheme 10

At pH 7.2, with all three metal ions, there is an apparent decrease in the number of protons released (Table 2) which may indicate that one of the protein ligands in the apo-enzyme, probably the protonated histidine in the 3H site, is partially dissociated.

Conclusions

The metallo-β-lactamase BcII forms both mono- and bi-nuclear species with native zinc and also with cobalt and cadmium. Which species is formed is controlled by the pH because some of the protein ligands used for metal-ion binding must lose a proton to form the metallo-enzyme from the apo-enzyme. At pH 5.2 and 5.6 the dominant species for all three metals is the mono-nuclear MBL, but only with the native mono-nuclear ZnBcII is the metal-bound water ionised. Compared with ZnBcII, fewer protons are released upon formation of mono-nuclear CoBcII and CdBcII which probably involves metal ion binding to the

DCH site. For all three metal-ions, the number of metal-ions binding per molecule of enzyme increases from one to two as the pH is increased from pH 5.20 to 7.20. Only cadmium shows distinct sequential binding in the ITC outputs and the two cadmium ions bind independently and non-cooperatively, showing very distinct and different binding parameters. The binuclear Cd₂BcII is formed with the release of only two protons from the apo-enzyme and probably has an unionised water bridging the two metal-ions, whereas in Zn₂BcII and Co₂BcII it is a bridged hydroxide-ion. The apparent single ITC titration curves when two zinc- or cobalt-ions are bound to the β-lactamase are not indicative of strong cooperative binding. The inhibition of the metallo-β-lactamase BcII by thiol peptides requires two zinc-ions in the active site.

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Notes and references

IPOS, The Page Laboratories, Department of Chemical and Biological Sciences, The University of Huddersfield, Queensgate, Huddersfield, HD1 3DH, United Kingdom.

Electronic Supplementary Information (ESI) available: [details of the cubic equation analysis and applications to ITC outputs for sequential and positive cooperative binding, ITC experimental data]. See DOI: 10.1039/b000000x/

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