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The Activity of the Dinuclear Cobalt- β -Lactamase from *Bacillus cereus* in Catalysing the Hydrolysis of β -Lactams

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Short title: The activity of dinuclear cobalt β -lactamase

Abbreviations: MBL, metallo- β -lactamases; BcII, *Bacillus cereus*- β -lactamase; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; PAC, perturbed angular correlation; LMCT, ligand to metal charge transfer; MCD, magnetic circular dichroism.

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ABSTRACT

Metallo- β -lactamases are native zinc enzymes that catalyse the hydrolysis of β -lactam antibiotics, but are also able to function with cobalt (II) and require one or two metal-ions for catalytic activity. The hydrolysis of cefoxitin, cephaloridine and benzylpenicillin catalysed by cobalt substituted β -lactamase from *Bacillus cereus* (BcII) has been studied at different pHs and metal-ion concentrations. An enzyme group of pK_a 6.52 ± 0.1 is found to be required in its deprotonated form for metal-ion binding and catalysis. The species that results from the loss of one cobalt ion from the enzyme has no significant catalytic activity and is thought to be the mono-nuclear CoBcII. It appears that di-nuclear CoBcII is the active form of the enzyme necessary for turnover, while the mono-nuclear CoBcII is only involved in substrate binding. The cobalt enzyme is a more efficient catalyst for the hydrolysis of some β -lactam antibiotics suggesting that the role of the metal-ion is predominantly to provide the nucleophilic hydroxide, rather than to act as a Lewis acid to polarise the carbonyl group and stabilise the oxyanion tetrahedral intermediate.

Introduction

The major bacterial defence mechanism against the normally lethal action of β -lactam antibiotics consists of bacteria producing a class of enzymes, the β -lactamases, which catalyse the hydrolysis of the β -lactam ring, rendering the drugs inactive (**Scheme 1**). From the mechanistic point of view, β -lactamases are divided into two classes: serine β -lactamases, (classes A, C and D β -lactamases), which use an active site serine residue for hydrolysis, and metallo- β -lactamases, (MBLs), (class B β -lactamases), which require one or two zinc ions for catalytic activity [1]. MBLs have been further divided into three subclasses, B1, B2 and B3, based on their amino acid sequences, substrate profiles and metal-ion requirement [2]. Subclass B1 of MBLs is the largest and contains four well-studied β -lactamases: BcII from *Bacillus cereus* [3-5], CcrA from *Bacteroides fragilis* [6-9], IMP-1 from *Pseudomonas aeruginosa* [10-12] and BlaB from *Chryseobacterium meningosepticum* [13]. They efficiently hydrolyze a wide range of substrates, including penicillins, cephalosporins and carbapenems [14]. The most common representatives of subclass B2 are CphA from *Aeromonas hydrophila* [15] and ImiS from *Aeromonas veronii* [16], which preferentially hydrolyze carbapenems, e.g., imipenem and meropenem [17], and have poor activity against penicillins and cephalosporins [18, 19]. Finally, subclass B3 contains the only known tetrameric zinc β -lactamase, the L1 enzyme from *Stenotrophomonas maltophilia* [20], and the monomeric FEZ-1 from *Legionella gormanii* [21]. Both enzymes hydrolyze a wide spectrum of β -lactam antibiotics [18, 19].

There are two zinc binding sites [2-4,6,12,13] but their occupancy has different effects in different MBLs. Furthermore, the zinc ligands in the two sites are not the same and are not fully conserved between the different MBLs. In the B1 enzymes, such as the *Bacillus cereus* MBL, BcII, (Figure 1) the zinc in site 1 (the histidine site or His₃ site) is four-coordinated through the nitrogens of the imidazoles of three histidine residues (116, 118, and 196) and a water molecule, Wat₁. The zinc in the second site (site 2, the DCH or the Cys site) is coordinated to five ligands - His263, Asp120, Cys221 and one water molecule, carbonate [22] or another water, often referred to as the apical water, or Wat₂ [3,6,23,24]. The two metal-ions are relatively close to each other, but the distance between them varies from 3.4 to 4.4 Å in different structures of the BcII and CcrA enzymes [3,6, 22-24]. The CcrA enzyme shows a bridging water ligand between the two metals that is thought to exist as a hydroxide ion [6, 24] as does BcII at pH 7.5 [25] although at lower pH this solvent molecule is strongly associated to the zinc in site 1 [3, 26].

MBLs have different metal-ion affinities for the two binding sites and apparently different metal-ion requirement for catalysis. The class B1 enzyme BcII, from *Bacillus cereus*, has very different dissociation constants for the two metal binding sites, based on fluorescence spectroscopy using a chromophoric chelator. For the loss of metal-ion from the mono-nuclear enzyme, the dissociation constant, K_{mono} , is $6.2 \times 10^{-10} \text{M}$ and that for the loss of one metal-ion from the di-nuclear MBL, K_{di} , is $1.5 \times 10^{-6} \text{M}$ [27]. K_{mono} decreases significantly, from nM to pM, in the presence of substrate whereas K_{di} decreases by only two fold [28]. This suggested that the mono-zinc enzyme is responsible for the catalytic activity under physiological conditions, where the concentration of free Zn^{2+} is in the pM region [28]. Conversely, and despite the very close similarity with BcII, the class B1 enzyme CcrA, from *Bacteroides fragilis*, binds both zinc ions very tightly [29]. Kinetic studies of CcrA with nitrocefin have shown that only the di-nuclear species is active and that a previously observed ‘mono-zinc’ CcrA enzyme [7] was a mixture of the di-zinc and the apo (metal free) enzyme [30].

Based on the pH-dependence of $k_{\text{cat}}/K_{\text{m}}$ for the ZnBcII catalysed hydrolysis of benzylpenicillin and cephaloridine, and of the competitive inhibition constant (K_{i}) of BcII with a thiol inhibitor, it has been proposed that there are three catalytically important groups in the enzyme: two of pK_{a} 5.6 which were assigned to Asp-120 and the zinc bound water, and are required in their deprotonated form for activity, and one of pK_{a} 9.5 which is required in its protonated form for maximum activity [31]. Increasing the metal-ion concentration (from 10^{-6} to 10^{-3}M) had no effect on the rate constant at neutral pH but restored the enzyme activity at lower pHs ($\text{pH} \leq 5.5$), with a corresponding decrease of the apparent pK_{a} values (from 5.6 to 4.5). Decreasing the pH liberates metal-ion from the enzyme due to protonation of one of the metal ligands. In terms of the equilibrium between mono- and di- nuclear enzymes, binding of the second zinc ion lowers the pK_{a} s of the catalytically important groups on the acidic limb [31]. The proposed mechanism involves the metal bound hydroxide attacking the carbonyl to generate a mono-anionic tetrahedral intermediate, which is followed by proton abstraction by the Asp120 to give a di-anionic species (**Scheme 2**). It was suggested that the same aspartic acid residue then functions as proton donor, to facilitate C-N bond fission and opening of the β -lactam ring [31].

Changing the metal-ion in zinc enzymes gives the opportunity to explore the structural and mechanistic role of the metal [32-37]. The zinc of metallo β -lactamases, can be substituted with cadmium, cobalt and manganese to give catalytically active enzymes [25,38,39]. The metal environment of cobalt substituted metallo β -lactamases has been studied by UV-Vis spectroscopy, NMR and EPR [4, 29, 40-43]. The kinetic effect of cobalt substitution in BcII is to

make the progress curves for the hydrolysis of benzylpenicillin biphasic, with an initial burst followed by a transient to a steady-state rate [44, 45]. The size of the burst was greater than the concentration of enzyme, which led the authors to propose a branched kinetic hydrolysis pathway involving two enzyme-substrate intermediates, ES^1 and ES^2 , which we have recently reinterpreted as mono- and di-nuclear forms of the enzyme [46]. The pH-dependencies of the Michaelis-Menten constants k_{cat}/K_m and k_{cat} for the Co-BcII catalysed hydrolysis of benzylpenicillin were performed at saturating concentrations of cobalt ion [38]. However, given more recent studies [31], which have shown that the pK_a value of the ionisation on the acidic limb of the ZnBcII pH-rate profile is metal-ion concentration dependent, we have studied the pH-rate profiles for CoBcII at different cobalt ion concentrations. Cobalt has about 100 fold less affinity than zinc for the two binding sites of BcII, i.e., the dissociation constants are higher, which makes it easier from an experimental point of view, for studying the differences between the catalytic properties of the mono- and the di-nuclear forms of the enzyme.

Experimental

Materials

Reagents used in all kinetic experiments were analytical or an equivalent grade. Cephaloridine was supplied by GlaxoSmithKline and used without further purification. Buffers, benzylpenicillin, cefoxitin, Chelex 100 and $CoCl_2$ (99.9999%) were purchased from Sigma. Deionised ultra pure water (18 M Ω cm) was used for the preparation of buffers and other aqueous solutions. The buffers used were: acetate (pK_a 4.75), MES (pK_a 6.15), MOPS (pK_a 7.20), TAPS (pK_a 8.40), CHES (pK_a 9.2). Buffer solutions were prepared just prior to the experiment, and their ionic strength was kept constant by means of potassium chloride.

The apo *Bacillus cereus* 569/H/9 enzyme (metal free BcII) was prepared by the following procedure: ZnBcII (prepared and purified as described [47]) was dialyzed, whilst stirring, against two changes of 0.015M MES, pH=6.5, containing 0.1M NaCl and 0.02M EDTA over a 12h period; EDTA was removed from the resulting apoenzyme solution by four dialysis steps against the same buffer containing 1M NaCl and Chelex 100 and finally two dialysis steps against 0.015M MES, pH=6.5 containing 0.1M NaCl and Chelex 100. The resulting apoenzyme contained less than 3% Zn^{2+} , as determined by atomic absorption spectroscopy, and less than 10% free EDTA, as shown by 1H -NMR.

Equipment

pH Measurements were made using a ϕ 40 pH meter (Beckman, Fullerton, U.S.A.) with a calomel glass electrode (Beckman). A two point calibration of the pH meter was taken at 30⁰C prior to use, with a pH 7 phosphate “green” buffer (Beckman) and a pH 4 or pH 10 calibration buffer (BDH, Poole, U.K.). UV spectrometry was carried out on a Cary 1E UV-Visible spectrometer equipped with a twelve compartment cell block thermostated by using a peltier system (Varian, Australia). Rate constants were estimated using the Cary Win UV kinetics application Version 02.00(26).

The concentration of free EDTA in the apo-enzyme solution was determined by ¹H-NMR spectra on a 400MHz instrument (Bruker, Germany), using the standard addition method.

The residual zinc content of apoBcII was determined by atomic absorption spectroscopy on a Perkin Elmer AAnalyst 100 atomic absorption spectrometer. The hollow cathode lamp wavelength was set at 213.9nm, with a current of 7mA, and a slit wave of 0.7nm. Zinc sulphate solutions of different concentrations were used as standards.

General kinetic procedure

In a typical experiment, the apoBcII (1×10^{-7} - 2×10^{-6} M) was incubated for 5 min at 30⁰C in the buffer (2ml) containing the corresponding cobalt ion concentration (10^{-4} - 10^{-3} M), in a quartz cuvette (200-2500nm, Hellma). Unless otherwise specified, the reaction was initiated by adding the substrate and was followed by the decrease in absorbance at 235nm for benzylpenicillin ($\Delta\epsilon=820\text{M}^{-1}\text{cm}^{-1}$) and 260nm for cefoxitin ($\Delta\epsilon=4000\text{M}^{-1}\text{cm}^{-1}$) and cephaloridine ($\Delta\epsilon=8000\text{M}^{-1}\text{cm}^{-1}$).

Steady-state kinetics

The Michaelis-Menten kinetic parameters, k_{cat} , K_{m} and $k_{\text{cat}}/K_{\text{m}}$ were determined as follows. Below saturation, where $[S] \ll K_{\text{m}}$, the curves were fitted to a simple first-order rate law to obtain the pseudo-first-order rate constants, k_{obs} , which were shown to be first-order in enzyme concentration. The second-order rate constant $k_{\text{cat}}/K_{\text{m}}$ was obtained by dividing k_{obs} by the concentration of enzyme used. For the determination of k_{cat} and K_{m} , the substrate concentrations used were in the range of the K_{m} values. The initial rates, measured for at least seven substrate concentrations, were fitted directly to the Michaelis Menten equation using SCIENTIST software (Micro Math Scientific Software, Utah, USA), to obtain the apparent k_{cat} and K_{m} values for each pH and each metal-ion concentration.

Results and Discussion

pH-rate profiles for Co-BcII catalysed hydrolysis of cefoxitin, cephaloridine and benzylpenicillin.

The kinetic studies of metallo-enzymes that involve the replacement of the native-enzyme should ensure that the contribution from any remaining wild-type enzyme to the measured activity is as small as possible. In this study, a background hydrolysis rate of all substrates (cefoxitin **(1)**, cephaloridine **(2)** and benzylpenicillin **(3)**) in the presence of the apo-enzyme was always determined and was insignificant, unless otherwise stated. In the following experiments, two cobalt ion concentrations were used, 10^{-3}M and 10^{-4}M , which should ensure that the mono-cobalt species is always present and that a variable di-cobalt species could be present as the dissociation constants for loss of the metal-ion from the mono- (K_{mono}) and di-cobalt (K_{di}) species are $9.3 \times 10^{-8}\text{M}$ and $6.0 \times 10^{-5}\text{M}$, respectively [27]. Under our experimental conditions at 30°C only clean first or zero order kinetics were observed over the entire progress curves for the hydrolysis of the substrates. The biphasic behaviour described in the introduction is only observed at low temperatures or low metal-ion concentrations [44-46]. For the three substrates studied here, cefoxitin **(1)**, cephaloridine **(2)** and benzylpenicillin **(3)**, the Michaelis constant, K_{m} , is much lower for the cobalt substituted enzyme than for the native zinc enzyme. This makes it difficult to obtain kinetic data below saturation conditions in order to determine K_{m} and $k_{\text{cat}}/K_{\text{m}}$, especially at lower pHs, as K_{m} decreases with decreasing pH.

Cefoxitin (1)

The catalytic constant, k_{cat} , for CoBcII catalysed hydrolysis of cefoxitin varies with pH and cobalt ion concentration as shown in **Figure 2**, indicative of activity being dependent on an ionisable group. Between pH 8 and 9 the reaction rate is pH-independent, but decreases at lower pHs. In the pH-rate dependent region, k_{cat} decreases with a first-order dependence on hydronium ion concentration, i.e., the slope of $\log k_{\text{cat}}$ against pH is unity. At low pH a 10 fold decrease in cobalt ion concentration decreases k_{cat} by approximately 10 fold, whereas in the pH-rate independent region k_{cat} changes less than three fold. The kinetically important ionisation in the k_{cat} -pH profile has an apparent $\text{p}K_{\text{a}}$ value of 7 and 6.5 for CoBcII catalysed hydrolysis of cefoxitin at 10^{-4} and 10^{-3}M CoCl_2 , respectively (**Figure 2**).

Although the Michaelis constant, K_{m} , could not be determined very accurately due to its very low value, especially at low pH, to a first approximation K_{m} is also pH-independent above pH 8

and there is an evident trend of K_m decreasing with decreasing the pH (**Figure 3**). The apparent sigmoidal dependence of pK_m on pH generates apparent pK_a values of 7.2 and 6.5, at $10^{-4}M$ and $10^{-3}M$ $CoCl_2$, respectively. The values of K_m may also be dependent on the concentration of metal-ion but the data is not definitive. At pH 8-9 and 5-6 there is little or no dependence on cobalt ion concentration but, between pH 6 and 7, K_m does vary about 6-7 fold when the concentration of $CoCl_2$ varies from $10^{-4}M$ to $10^{-3}M$ (**Figure 3**).

Similarly, the values of the second-order rate constant, k_{cat}/K_m , for the CoBcII catalysed hydrolysis of cefoxitin, are subject to considerable error (due to the errors involved in determining the K_m) but show an apparent bell shape pH-rate profile, with a decrease in rate at high and low pH and an intermediate pH-independent region. The rate constant shows a first-order dependence on both metal-ion and acid concentration at low pH, whereas in the pH-independent region, k_{cat}/K_m increases by less than 2 fold for a 10 fold increase in metal-ion concentration. The apparent pK_a values for the acidic ionisation in the pH- k_{cat}/K_m profile are 6.3 and 5.8 for 10^{-4} and $10^{-3}M$ $CoCl_2$, respectively.

Cephaloridine (2)

Using cephaloridine as a substrate, a similar trend for the variation of k_{cat} , (**Figure 4**), K_m and k_{cat}/K_m (data not shown) with pH at different $CoCl_2$ concentrations was observed. At low pH, the log k_{cat} versus pH profile shows a first-order decrease of k_{cat} with increasing hydronium ion concentration, which is again metal-ion concentration dependent, with apparent pK_a values of 6.8 and 6.2 for 10^{-4} and $10^{-3}M$ $CoCl_2$, respectively (**Figure 4**). The rate increases about 6 fold at low pH and less than 2 fold in the pH-independent region with increasing the $CoCl_2$ concentration from 10^{-4} to $10^{-3}M$.

The decrease of K_m with decreasing pH also appears metal-ion concentration dependent, and K_m varies from about 3×10^{-5} and $5 \times 10^{-5}M$ at high pHs to about 3×10^{-6} and $8 \times 10^{-6}M$ at low pHs, at 10^{-4} and $10^{-3}M$ $CoCl_2$, respectively. Whilst the quality of the data is poor, (due to the difficulties of attaining below saturation conditions), it is possible to see that the second-order rate constant, k_{cat}/K_m , decreases at pHs less than 6 and is metal-ion concentration independent in the pH-independent region. Unlike cefoxitin, there is no obvious evidence of a decrease in k_{cat}/K_m at higher pH for cephaloridine.

Benzylpenicillin (3)

For CoBcII catalysed hydrolysis of benzylpenicillin only the k_{cat} versus pH profile has been determined at different metal-ion concentrations ($[\text{Co}^{2+}] = 10^{-3}\text{M}$ and 10^{-4}M), (**Figure 5**), due to the very low K_{m} values ($<10^{-4}\text{M}$). At lower pHs, k_{cat} decreases with decreasing metal-ion concentration, with apparent pK_{a} values of 6.8 and 6.2 for 10^{-4}M and 10^{-3}M CoCl_2 , respectively. As with the other substrates, at low pH, k_{cat} shows a first-order dependence on acid and on metal-ion concentration, while in the pH-independent region, k_{cat} increases by less than 2 fold on increasing the CoCl_2 concentration from 10^{-4} and 10^{-3}M (**Figure 5**).

Mechanistic models

The variation of k_{cat} and $k_{\text{cat}}/K_{\text{m}}$ with pH and cobalt ion concentration is similar for the three substrates used, which indicates that the phenomena are an intrinsic property of the enzyme and independent of the nature of the substrate. The decrease in the apparent pK_{a} values with increasing cobalt ion concentration suggests that the inverse first-order effect of the acid concentration on the rate of hydrolysis at lower pHs is due to the protonation of a metal ligand, which is responsible for the loss of one cobalt ion from the enzyme active site, rather than to the protonation of a catalytically important group or to a change in the rate limiting step. The decrease in the values of k_{cat} and K_{m} with decreasing pH and metal-ion concentration suggests that there are at least two enzyme species, one of which has greater values of k_{cat} and K_{m} , and is dominant at high pH and metal-ion concentration. The other species results from the loss of a cobalt ion and has a lower K_{m} and k_{cat} , and is probably, effectively, inactive. Several possible explanations for the metal-ion concentration and pH-dependencies have been considered (**Scheme 3**).

In the model (i), the apo-enzyme is the dominant species present at low pH and low metal-ion concentration and is catalytically inactive, while the mono-cobalt enzyme is responsible for the catalytic activity. Even though this model explains the variation of k_{cat} and $k_{\text{cat}}/K_{\text{m}}$ with pH and metal-ion concentration, it does not explain the decrease in K_{m} with decreasing pH (tighter substrate binding) as it has been shown that the apoenzyme does not bind the substrate, at least in the case of benzylpenicillin and some cephalosporins [48]. It follows that the enzyme species involved in the tight substrate binding (low K_{m}), at low pH and metal-ion concentration, is probably the mono-cobalt BcII. The observed rate dependence on cobalt ion concentration can be explained if the catalytically active species is formed by the uptake of one cobalt ion *i.e.* is a di-cobalt enzyme species (models (ii), (iii) or (iv)). The increase in rate with increasing pH can be

explained by the ionisation of a group involved in the binding of the second cobalt ion: this can be either the metal bound water (model ii) or a protein residue, in which case the bridging water can be either deprotonated (model iii) or protonated (model iv). The interconversion between mono- and di-cobalt enzyme species is also supported by the biphasic kinetics observed at lower temperatures [46].

A more detailed pathway involves the mono-cobalt deprotonated (ECo) enzyme, which forms at higher pHs, in substrate binding and possibly catalysis (**Scheme 4**). The experimental data for the three substrates were fitted to the equations (1)-(2), which describe the variation of k_{cat} and K_m with pH and metal-ion concentration, according to **Scheme 4**. The data for the pK_m -pH and $\log k_{cat}$ -pH dependencies were fitted to equations (1) and (2) using the literature value of K_{di} for K_d , $6.0 \times 10^{-5} M$ [27], in order to obtain the substrate dependent parameters, K_m , K_{m1} and K_{m2} , k_{cat} , k_{cat1} and k_{cat2} and the substrate independent parameter, K_a . It was found that the k_{cat} values for the mono-cobalt enzyme, in both the protonated and deprotonated forms, (k_{cat1} and k_{cat2} , respectively), were close to zero ($< 0.1 s^{-1}$). The only catalytically active species is the di-cobalt enzyme. For benzylpenicillin, for which only the values of k_{cat} were available, the data were fitted to equation (1), fixing the value for K_m as $1.6 \times 10^{-4} M$. The derived constants for the three substrates are summarized in **Table 1**. The pK_a value for the kinetically important ionisation was found to be 6.52 ± 0.1 . This pK_a may correspond either to the cobalt bound water (hydroxide ion), whose protonation results in dissociation of a cobalt ion from the enzyme active site, or to a metal binding enzyme residue, such as Cys221. The pK_a of Cys 221 (or Cys221-His263 pair) in the apoBcII was found to be 7.85 [46], but may decrease in the mononuclear species.

$$k_{cat}^{obs} = \frac{\frac{k_{cat1}}{K_{m1}} + \frac{k_{cat2}}{K_{m2}} \cdot \frac{K_a}{[H^+]} + \frac{k_{cat}}{K_m} \cdot \frac{K_a}{[H^+]} \cdot \frac{[Co^{2+}]}{K_d}}{\frac{1}{K_{m1}} + \frac{1}{K_{m2}} \cdot \frac{K_a}{[H^+]} + \frac{1}{K_m} \cdot \frac{K_a}{[H^+]} \cdot \frac{[Co^{2+}]}{K_d}} \quad (1)$$

$$K_m^{obs} = \frac{1 + \frac{K_a}{[H^+]} + \frac{K_a}{[H^+]} \cdot \frac{[Co^{2+}]}{K_d}}{\frac{1}{K_{m1}} + \frac{1}{K_{m2}} \cdot \frac{K_a}{[H^+]} + \frac{1}{K_m} \cdot \frac{K_a}{[H^+]} \cdot \frac{[Co^{2+}]}{K_d}} \quad (2)$$

It is commonly suggested that the role of the metal-ion in metalloproteases is to act as a Lewis acid by co-ordination to the peptide carbonyl oxygen giving a more electron deficient carbonyl carbon which then facilitates nucleophilic attack and stabilises the negative charge developed on the carbonyl oxygen of the tetrahedral intermediate anion. Another role of the metal-ion is to lower the pK_a of the co-ordinated water so that the concentration of metal-bound 'hydroxide ion' is increased relative to bulk solvent hydroxide ion at neutral pH. This metal-bound conjugate base is also a better nucleophile than water, but obviously not better than 'free' hydroxide ion. The dissociation of an acid with a low pK_a generates a weaker base which usually corresponds to a weaker nucleophile. Hence the order of basic strength and so nucleophilicity is: aqueous hydroxide-ion, metal-bound hydroxide-ion then water. The better is the Lewis acid (*i.e.* a more electron deficient metal-ion centre) in stabilising the negative charge developed on the tetrahedral intermediate from the β -lactam carbonyl oxygen, the greater will also be the effect on the acidity of bound water *i.e.* the lower the pK_a . However, a lower pK_a of zinc-bound water gives rise to a more weakly nucleophilic hydroxide-ion even though it may become the dominant species even at lower pH. Conversely, a weaker Lewis acid would be less effective at reducing the pK_a of the zinc bound water but, at pHs above the pK_a , would produce a more nucleophilic hydroxide-ion. Similarly a high pK_a water implies a weak Lewis acid and so the zinc ion will be less efficient at stabilising the tetrahedral intermediate. The main factors controlling the charge density on metal-ions with the same formal positive charge are ionic radius and the number and type of its ligands. Substitution of the native zinc in the metallo- β -lactamases by other metal-ions enables an exploration of different Lewis acidities on catalytic activity.

A comparison of the pH - k_{cat}/K_m profiles for cobalt substituted BcII species with that of the native zinc enzyme [31], for the hydrolysis of cefoxitin and cephaloridine, shows the cobalt enzyme to have a higher maximum activity at their pH optimum and to be more active than ZnBcII at pH 7. The cobalt enzyme is remarkably about 200 and 10 fold more active than the native ZnBcII with cefoxitin and cephaloridine as a substrate, respectively (Table 1). *Assuming* that the pK_a of the cobalt bound water is 6.5 and that of the zinc bound water is 5.6 in BcII [31], then Co^{2+} has a higher effective positive charge, so it is a weaker Lewis acid than Zn^{2+} , but it gives a more nucleophilic metal-bound hydroxide ion. At pH 7, 96% of ZnBcII and 76% of CoBcII will be in their active deprotonated forms. The fact that the cobalt substituted BcII is more active than the native ZnBcII (Table 1) suggests that the role of the metal-ion is predominantly to provide the nucleophilic hydroxide, rather than to act as a Lewis acid to polarise the carbonyl group and stabilise the oxyanion tetrahedral intermediate.

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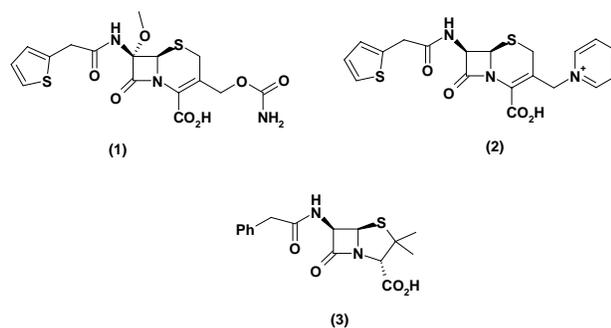
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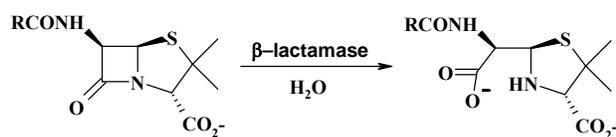
Table 1. Calculated values of the rate and dissociation constants from Scheme 4, for CoBcII catalysed hydrolysis of cefoxitin (**1**), cephaloridine (**2**) and benzylpenicillin (**3**) in buffer, 0.025M, I=0.25M, at 30°C, ($K_d=6.0 \times 10^{-5}$ M). The corresponding values, determined in similar conditions for the native ZnBcII, are given for comparison.

		k_{cat}/K_m ($10^6 \text{ M}^{-1} \text{ s}^{-1}$)	k_{cat} (s^{-1})	K_m (10^{-6} M)	pK_a	K_{m1} (10^{-6} M)	K_{m2} (10^{-6} M)
1	Co²⁺	0.052±0.009	16±2.0	310±10	6.52±0.1	12±1.0	100±20
	Zn²⁺	0.00027±0.00005	0.57±0.1	2100±200			
2	Co²⁺	1.7±0.3	67±10	40±10	6.52±0.1	3.0±0.3	25±5.0
	Zn²⁺	0.15±0.02	160±20	1060±150	5.5±0.05 ^a		
3	Co²⁺	2.4±0.4	390±20	160±20	6.52±0.1	12±2.0	500±100
	Zn²⁺	0.87±0.1	950±120	1100±200	5.8±0.05 ^a		

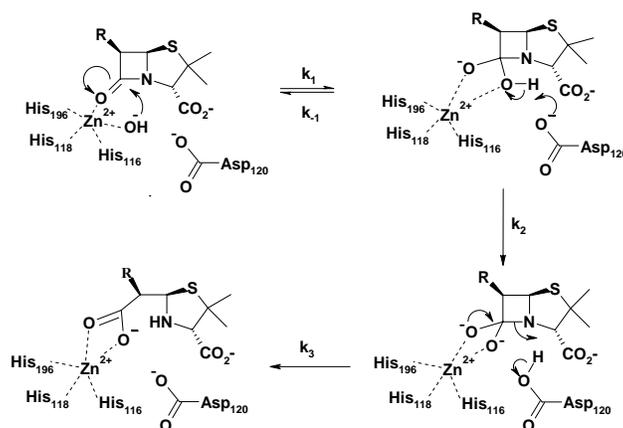
^a values taken from ref [31]



SCHEMES



Scheme 1



Scheme 2

FIGURES

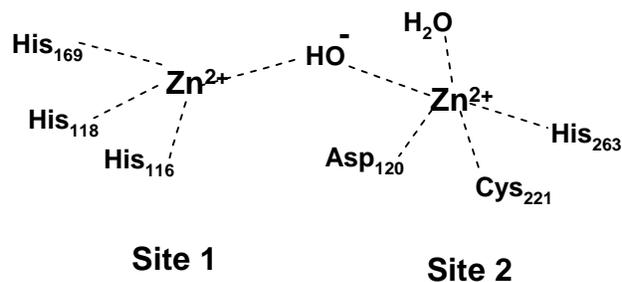


Figure 1. Schematic representation of the two zinc binding sites in the metallo- β -lactamase from *Bacillus cereus*, BcII.

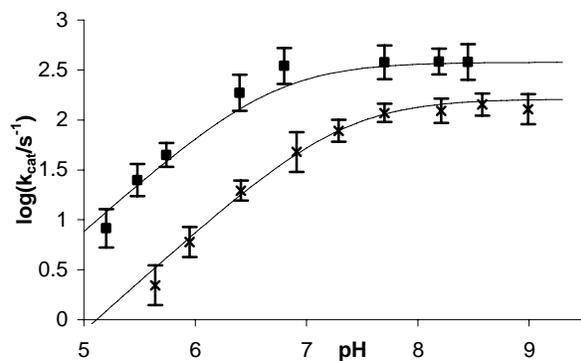


Figure 2. Plot of $\log k_{cat}$ against pH for CoBcII catalysed hydrolysis of cefoxitin in 0.025M buffer, $I=0.25M$, in presence of $10^{-4}M Co^{2+}$ (x) and $10^{-3}M Co^{2+}$ (■); the solid lines are the calculated values using equation (1) and the parameters in Table 1 (Scheme 4).

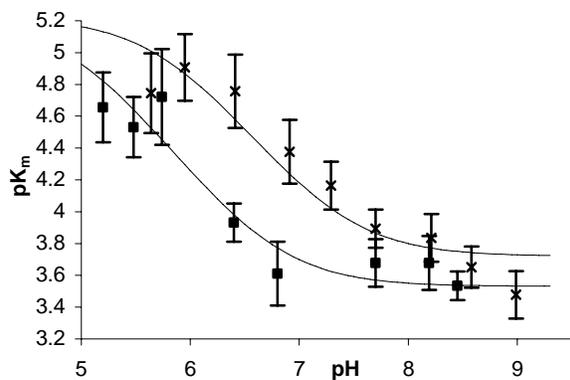


Figure 3. Plot of pK_m against pH for CoBcII catalysed hydrolysis of cefoxitin in 0.025M buffer, $I=0.25M$, in presence of $10^{-4}M$ Co^{2+} (x) and $10^{-3}M$ Co^{2+} (■); the solid lines are the calculated values using equation (2) and the parameters in Table 1 (Scheme 4).

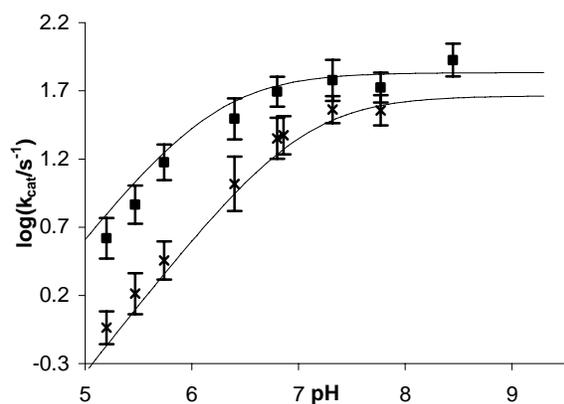


Figure 4. Plot of $\log k_{cat}$ against pH for CoBcII catalysed hydrolysis of cephaloridine in 0.025M buffer, $I=0.25M$, in presence of $10^{-4}M$ Co^{2+} (x) and $10^{-3}M$ Co^{2+} (■); the solid lines are the calculated values using equation (1) and the parameters in Table 1 (Scheme 4).

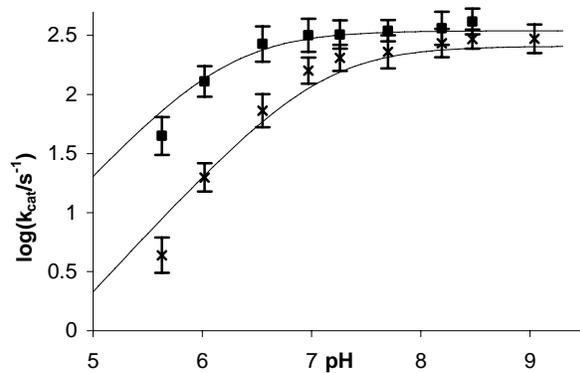


Figure 5. Plot of $\log k_{\text{cat}}$ against pH for CoBcII catalysed hydrolysis of benzylpenicillin in 0.025M buffer, $I=0.25\text{M}$, in presence of 10^{-4}M Co^{2+} (x) and 10^{-3}M Co^{2+} (■); the solid lines are the calculated values using equation (1) and the parameters in Table 1 (Scheme 4).