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The Variation of Catalytic Efficiency of *Bacillus cereus* Metallo- β -lactamase with Different Active Site Metal Ions[†]

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ABSTRACT: The kinetics and mechanism of hydrolysis of the native zinc and metal substituted *Bacillus cereus* (BcII) metallo- β -lactamase have been investigated. The pH and metal ion dependence of k_{cat} and $k_{\text{cat}}/K_{\text{m}}$, determined under steady-state conditions, for the cobalt substituted BcII catalyzed hydrolysis of cefoxitin, cephaloridine, and cephalixin indicate that an enzyme residue of apparent $\text{p}K_{\text{a}}$ 6.3 ± 0.1 is required in its deprotonated form for metal ion binding and catalysis. The $k_{\text{cat}}/K_{\text{m}}$ for cefoxitin and cephalixin with cadmium substituted BcII is dependent on two ionizing groups on the enzyme: one of $\text{p}K_{\text{a}1} = 8.7 \pm 0.1$ required in its deprotonated form and the other of $\text{p}K_{\text{a}2} = 9.3 \pm 0.1$ required in its protonated form for activity. The pH dependence of the competitive inhibition constant, K_{i} , for CdBcII with L-captopril indicates that $\text{p}K_{\text{a}1} = 8.7 \pm 0.1$ corresponds to the cadmium-bound water. For the manganese substituted BcII, the pH dependence of $k_{\text{cat}}/K_{\text{m}}$ for benzylpenicillin, cephalixin, and cefoxitin similarly indicated the importance of two catalytic groups: one of $\text{p}K_{\text{a}1} = 8.5 \pm 0.1$ which needs to be deprotonated and the other of $\text{p}K_{\text{a}2} = 9.4 \pm 0.1$ which needs to be protonated for catalysis; the $\text{p}K_{\text{a}1}$ was assigned to the manganese-bound water. The rate was metal ion concentration dependent at the highest manganese concentrations used (10^{-3} M). The metal substituted species have similar or higher catalytic activities compared with the zinc enzyme, albeit at pHs above 7. Interestingly, with cefoxitin, a very poor substrate for ZnBcII, both k_{cat} and $k_{\text{cat}}/K_{\text{m}}$ increase with increasing $\text{p}K_{\text{a}}$ of the metal-bound water, in the order $\text{Zn} < \text{Co} < \text{Mn} < \text{Cd}$. A higher $\text{p}K_{\text{a}}$ for the metal-bound water for cadmium and manganese BcII leads to more reactive enzymes than the native zinc BcII, suggesting that the role of the metal ion is predominantly to provide the nucleophilic hydroxide, rather than to act as a Lewis acid to polarize the carbonyl group and stabilize the oxyanion tetrahedral intermediate.

Metallo- β -lactamases (MBLs¹) are bacterial enzymes that require one or two zinc ions for the hydrolysis of β -lactam antibiotics (1). The first metallo- β -lactamase to be discovered in 1966 was produced by an innocuous strain of *Bacillus cereus*, but in the last 40 years, MBL-mediated resistance has appeared in several pathogenic strains and is being rapidly spread by horizontal transfer, involving both plasmid and integron-borne genetic elements (2). MBLs represent a great clinical threat to β -lactam antibiotic therapy as, presently, there is no clinically useful inhibitor for this class of β -lactamases. According to their amino acid sequences, substrate profile, and metal ion requirement, MBLs can be divided into three subclasses: B1, B2, and B3 (3). Subclass

B1 is the largest and contains four well-studied β -lactamases: BcII from *B. cereus* (4–6), CcrA from *Bacteroides fragilis* (7–10), IMP-1 from *Pseudomonas aeruginosa* (11–13), and BlaB from *Cryseobacterium meningosepticum* (14). They efficiently hydrolyze a wide range of substrates, including penicillins, cephalosporins, and carbapenems (15). The most common enzyme representatives of subclass B2 are CphA from *Aeromonas hydrophila* (16) and ImiS from *Aeromonas veronii* (17), which preferentially hydrolyze carbapenems, e.g., imipenem and meropenem (18), but have poor activity against penicillins and cephalosporins (19, 20). Finally, subclass B3 contains the only known tetrameric zinc β -lactamase, the L1 enzyme from *Stenotrophomonas maltophilia* (21) and the monomeric FEZ-1 from *Legionella gormanii* (22).

The structures of several MBLs have been determined by X-ray diffraction, and all-BcII (4, 23), CcrA (7, 24), IMP-1 (13), L1 (25), FEZ-1 (26), CphA (27), and BlaB (14)—show a similar $\alpha\beta\beta\alpha$ fold. The active site of MBLs is situated at the bottom of a wide shallow groove between two β -sheets and has two potential zinc ion binding sites at the active site often referred to as sites 1 and 2 (28–31). The zinc ligands in the two sites are not the same and are not fully conserved between the different MBLs. In the subclass B1 enzymes, such as the *B. cereus* enzyme, BcII, the zinc in site 1 (the histidine site or His₃ site) is tetracoordinated by

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¹ Abbreviations: MBLs, metallo- β -lactamases; BcII, *Bacillus cereus* metallo- β -lactamase; NMR, nuclear magnetic resonance; MES, 2-[N-morpholino]ethanesulfonic acid; MOPS, 3-[N-morpholino]propane-sulfonic acid; TAPS, N-tris[hydroxymethyl]methyl-3-aminopropane-sulfonic acid; CHES, 2-[N-cyclohexylamino]ethanesulfonic acid; HEPES, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; EDTA, ethylenediaminetetraacetic acid; EXAFS, extended X-ray absorption fine structure; PAC spectroscopy, perturbed angular correlation spectroscopy; ITC, isothermal titration calorimetry.

the imidazoles of three histidine residues (116, 118, and 196) and a water molecule, Wat₁. In site 2 (DCH or Cys site) the metal is pentacoordinated by His 263, Asp 120, Cys 221, and one water molecule; the fifth ligand at site 2 is carbonate (23) or water, often referred to as the apical water, or Wat₂ (4, 7, 24, 29). Wat₂ is missing in one structure (23) and also in structures with inhibitors bound (13). 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 (4, 7, 23, 24, 29). Several structures of the CcrA enzyme show a bridging water ligand between the two metals that is thought to exist as a hydroxide ion (7, 29). In a structure of BcII containing two zinc ions determined at pH 7.5 there is also a similar bridging water molecule (32), but in structures of this enzyme at lower pH this solvent molecule is strongly associated with the zinc in site 1 (4, 31).

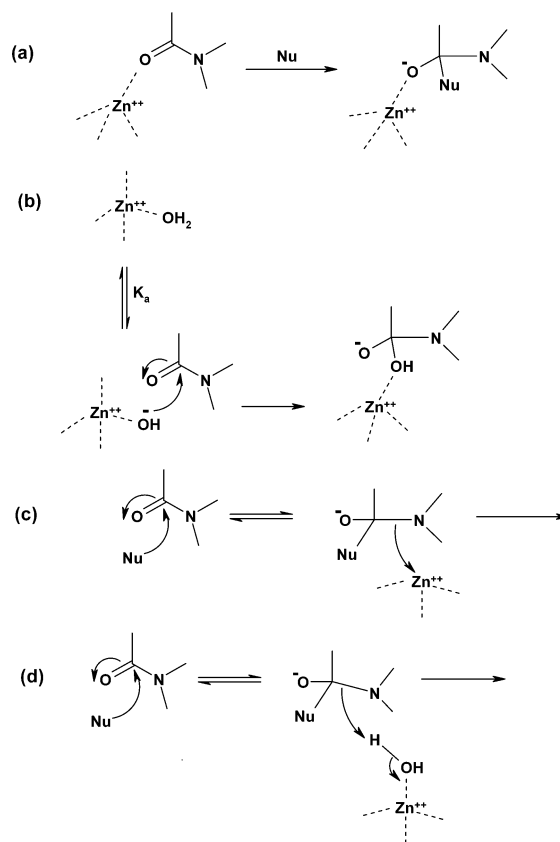
Although all MBLs have two conserved zinc binding sites, they have different metal ion affinities for the two binding sites and different metal ion requirements for catalysis. The BcII enzyme from *B. cereus* has been reported to have very different dissociation constants for the two metal binding sites. Although the first crystal structure, obtained at low pH (31), shows only one zinc ion bound to the histidine site, metal binding studies by fluorescence spectroscopy using a chromophoric chelator indicated a dissociation constant for the loss of zinc ion from the mononuclear enzyme, K_{mono} , of 6.2×10^{-10} M and one for the loss of one zinc ion from the dinuclear MBL, K_{di} of 1.5×10^{-6} M (33). Moreover, in the presence of substrate (imipenem), K_{mono} was found to decrease significantly, from nM to pM, while K_{di} decreased only by 2-fold (34). This suggested that the monozinc enzyme is responsible for the catalytic activity under physiological conditions, where the concentration of the free Zn^{2+} is in the pM region.

CcrA from *Bacteroides fragilis* binds both zinc ions very tightly (35). Despite the very close similarity with BcII, CcrA has a much higher affinity for the second zinc ion. In early kinetic studies of CcrA it was proposed that both the mono- and the dinuclear forms of the enzyme were catalytically active, with slightly different activities, at neutral pH (8). However, later studies have shown that only the dinuclear species is active and that the previously observed "monozinc" CcrA was a mixture of the dizinc and the apo (metal free) enzyme (36).

Class B2 metallo- β -lactamases appear to be catalytically active with one bound zinc ion, the binding of the second zinc ion noncompetitively inhibiting the enzyme, with a K_i of 5.0×10^{-5} M (16). The dissociation constant of the first zinc ion was found to be $(7.0 \text{ and } 1.2) \times 10^{-12}$ M in the absence and presence of substrate (imipenem), respectively (34). Although the metal ion requirement in MBL catalyzed hydrolysis of β -lactam antibiotics is still a matter of debate, catalytic mechanisms have been proposed for both the mono- and dinuclear enzymes.

There are many potential mechanistic roles for the metal ion in metallo-proteases (37), and they may well vary from enzyme to enzyme. It is commonly suggested that the metal ion acts as a Lewis acid by coordination to the peptide carbonyl oxygen, giving a more electron deficient carbonyl carbon, which facilitates nucleophilic attack (37, 38). The metal ion thus stabilizes the negative charge developed on

Scheme 1



the carbonyl oxygen of the tetrahedral intermediate anion (Scheme 1a). Many metallo-proteases have a water molecule directly coordinated to the metal ion which may act as the nucleophile to attack the carbonyl carbon (37, 38). The role of the metal ion is to lower the pK_a of the coordinated water so that the concentration of metal-bound hydroxide ion, albeit different, is increased relative to bulk solvent hydroxide ion at neutral pH and is a better nucleophile than water (Scheme 1b). Several mechanisms proposed for MBLs have incorporated these features (7, 31, 39). Although C–N bond fission is the most energetically difficult process in peptide hydrolysis, little attention is normally given to the mechanism of the breakdown of the tetrahedral intermediate. Breakdown of the tetrahedral intermediate could be facilitated by direct coordination of the departing amine nitrogen to the metal ion (Scheme 1c). This is the mechanism adopted for the zinc ion catalyzed hydrolysis of penicillin in aqueous solution (40). Alternatively, a metal-bound water could act as a general acid catalyst protonating the amine nitrogen leaving group to facilitate C–N bond fission (7) (Scheme 1d). Despite intense mechanistic studies, the detailed roles of the metal ion in metallo-proteases remain controversial (38) and distinguishing between the relative importance of the possible roles for zinc is complex.

The effective positive charge on the zinc ion depends on the number and nature of its ligands. Coordination to zinc of a ligand, with an ionizable hydrogen, lowers its pK_a , and the ionized ligand obviously is better at neutralizing the positive charge density on the metal. The pK_a of water bound to zinc in aqueous solution is 9.0, but is there evolutionary pressure to lower the pK_a of the zinc-bound water in an enzyme? This could be achieved, for example, by replacing, say, a negatively charged carboxylate ligand by a neutral

Table 1: Dissociation Constants for the His₃ (K_{mono}) Site and DCH (K_{di}) Site of *B. cereus* 569/H/9 Metallo- β -lactamase in 15 mM HEPES, 0.2 M NaCl, pH = 7.0⁵⁷ (33)

	Zn(II)	Cd(II)	Co(II)	Mn(II)
K_{mono} (M)	$(6.2 \pm 0.8) \times 10^{-10}$	$(8.3 \pm 0.5) \times 10^{-9}$	$(9.3 \pm 1.5) \times 10^{-8}$	
K_{di} (M)	$(1.5 \pm 0.7) \times 10^{-6}$	$(5.9 \pm 1.0) \times 10^{-6}$	$(6.6 \pm 1.0) \times 10^{-5}$	$> 10^{-3}$

histidine. Changing zinc-bound histidine for aspartate in carbonic anhydrase increases the pK_a of the zinc-bound water from 6.8 to ≥ 9.6 (41). Does a higher or lower pK_a metal-bound water lead to a faster reaction and more efficient catalysis? A low pK_a implies a more electron deficient metal ion center, which would give a better Lewis acid to stabilize the negative charge developed on the oxyanion of the tetrahedral intermediate. Similarly, a high pK_a for the metal-bound water implies a weaker Lewis acid, and so the zinc ion will be less efficient at stabilizing the tetrahedral intermediate. Conversely, the lower the pK_a of metal-bound water, the more “tightly bound” and stabilized is the resulting hydroxide ion, which, although it becomes the dominant species even at low pH, corresponds to a more weakly nucleophilic hydroxide ion. For example, if the pK_a of the zinc-bound water is about 5, then the nucleophilicity of the metal-bound hydroxide ion is only similar to that of a carboxylate anion. If a major role of the metal ion is to provide a better nucleophile than water, then the net effect depends on the relative importance of concentration and the dependence of the rate upon nucleophilicity. If the pK_a is “too high”, metal-coordinated water will be the dominant species over the desired pH range, but deprotonation will give a more nucleophilic metal-bound hydroxide. How reactivity changes with changing pK_a and pH will depend on the susceptibility of the rate of reaction to the basicity of the nucleophile—the hydroxide ion bound to the metal—as indicated by the Bronsted β_{nuc} value.

The simplest way to modify the pK_a of the zinc-bound water is to change the ligands or the metal ion. If the activity of the resulting enzyme is *inversely* proportional to the pK_a of the zinc-bound water ligand, this would be compatible with the zinc coordinating to the carbonyl oxygen and stabilization of the negative charge developed on this oxygen following nucleophilic attack. The lower pK_a of zinc-bound water indicates a more electrophilic zinc, which is better at stabilizing negative charge, giving rise to a better catalyst. Stabilization of the intermediate anion must be more important than the nucleophilicity of the zinc hydroxide. Conversely, if activity increases with basicity, i.e. with increasing pK_a , then this could indicate a greater role for nucleophilicity of the zinc hydroxide ion compared with the Lewis acid role of the metal ion stabilizing the negative charge development on the carbonyl oxygen. Substitution of the native zinc in the metallo- β -lactamases by other metal ions enables an exploration of the electronic and geometric structure of the active site and thus provides insights into the mechanism of action of the enzyme. For the metallo- β -lactamase from *B. cereus* (BcII), the Co, Cd, and Mn substituted species show significant catalytic activity for the hydrolysis of β -lactam antibiotics (32, 42, 43). The affinity of the metal for the two binding sites in BcII decreases in the series Zn > Cd > Co > Mn (Table 1) (33). The difference between the dissociation constants of the first and second binding sites is similar: about 3 orders of magnitude

for Zn, Cd, and Co. It has been shown that the zinc dissociation constants in BcII decrease, i.e., the metal ion is bound more tightly, in the presence of substrate (34).

In order to see the influence of the Lewis acidity of the metal ion on the catalytic efficiency of BcII, the native zinc was replaced by other transition metal ions: cobalt(II), cadmium(II), and manganese(II), with Lewis acidities increasing in the series $\text{Cd}^{2+} < \text{Mn}^{2+} < \text{Co}^{2+} < \text{Zn}^{2+}$. It is of interest to determine the pK_a of the metal-bound water in cobalt, cadmium, and manganese BcII, compare it with that of the zinc-bound water, and correlate these pK_a s with the hydrolytic activities of the corresponding metalloenzyme species. BcII 569/H/9 metallo- β -lactamase from *B. cereus* is a special case of MBL, since it has very different affinities for the first and second zinc ions, but very similar reported catalytic efficiencies of the mono- and dinuclear enzyme species. As different metal ions have different affinities for the BcII active site, metal ion substitution was also explored as a probe for discriminating between the catalytic activities of the mono- and dinuclear enzyme species.

EXPERIMENTAL PROCEDURES

Materials. Reagents used in all kinetic experiments were analytical grade or an equivalent grade. Cephaloridine was supplied by Glaxo Smith Kline and used without further purification. Buffers, benzylpenicillin, cefoxitin, cephalixin, Chelex 100, and the metal ion salts (CoCl_2 , CdCl_2 , MnCl_2 , and ZnSO_4 99.9999%) were purchased from Sigma. D_2O , NaOD, and DCl were obtained from Goss Scientific Ltd. Deionized ultrapure 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), and 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 metallo- β -lactamase (BcII from *B. cereus* 569/H/9) was supplied as an aqueous suspension in 10 mM HEPES buffer by Dr. Christian Damblon (University of Leicester, U.K.). The apo *B. cereus* 569/H/9 enzyme (the metal free BcII) was prepared by the following procedure: ZnBcII was dialyzed against two changes of 0.015 M MES, pH = 6.5, containing 0.1 M NaCl and 0.02 M EDTA over a 12 h period with stirring; EDTA was removed from the resulting apo-enzyme solution by four dialysis steps against the same buffer containing 1 M NaCl and Chelex 100 and finally two dialysis steps against 0.015 M MES, pH = 6.5 containing 0.1 M NaCl and Chelex 100. The resulting apo-enzyme 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 $\phi 40$ pH meter (Beckman, Fullerton, CA) with a calomel glass electrode (Beckman). A two point calibration of the pH meter was taken at 30 °C prior to use, with a pH 7 phosphate “green” buffer (Beckman) and a pH 4 or pH 10 calibration buffer (BDH, Poole, U.K.). pD values were taken as pH meter readings + 0.40.

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 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 213.9 nm, with a current of 7 mA, and a slit wave of 0.7 nm. Zinc sulfate solutions of different concentrations were used as standards.

General Kinetic Procedure. In a typical experiment, the apoBcII (1×10^{-7} to 2×10^{-6} M) was incubated for 5 min at 30 °C in the buffer (2 mL) containing the corresponding metal ion concentration (10^{-7} – 10^{-3} M), in a quartz cuvette (200–2500 nm, Hellma). Unless otherwise specified, the reaction was initiated by adding the substrate and was followed by the decrease in absorbance at 235 nm for benzylpenicillin ($\Delta\epsilon = 820 \text{ M}^{-1} \text{ cm}^{-1}$) and 260 nm for cefoxitin ($\Delta\epsilon = 4000 \text{ M}^{-1} \text{ cm}^{-1}$), cephalixin ($\Delta\epsilon = 7000 \text{ M}^{-1} \text{ cm}^{-1}$), and cephaloridine ($\Delta\epsilon = 8000 \text{ M}^{-1} \text{ cm}^{-1}$).

The Michaelis–Menten kinetic constants, 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), to obtain the apparent k_{cat} and K_{m} values for each pH and each metal ion concentration.

In all cases, the observed rate of hydrolysis in the absence of metal ion (apo-enzyme in metal free buffer) was negligible (less than 5%) compared to that in the presence of the metal ion.

Inhibition Studies. The second-order rate constants for the hydrolysis of cephalixin (1×10^{-4} M) catalyzed by CdBcII ([apoBcII] = 1×10^{-7} to 2×10^{-7} M, $[\text{Cd}^{2+}] = 1 \times 10^{-4}$ M), at different inhibitor (L-captopril) concentrations (1×10^{-6} to 1×10^{-5} M), were determined from initial rates. The inhibition constant of CdBcII by L-captopril, K_{i} , was determined using eq 1. Below saturation, this can be written in the form of eq 2, from which a plot of the inverse of the second-order rate constant, $(K_{\text{m}}/k_{\text{cat}})_{\text{I}}$, against $[I]$ gives the intercept on the inhibitor concentration axis equal to $-K_{\text{i}}$.

$$\text{initial rate} = \frac{[E][S]k_{\text{cat}}}{[S] + K_{\text{m}}\left(1 + \frac{[I]}{K_{\text{i}}}\right)} \quad (1)$$

$$\left(\frac{K_{\text{m}}}{k_{\text{cat}}}\right)_{\text{I}} = \left(\frac{K_{\text{m}}}{k_{\text{cat}}}\right)_0 + \left(\frac{K_{\text{m}}}{k_{\text{cat}}}\right)_0 \left(1 + \frac{[I]}{K_{\text{i}}}\right) \quad (2)$$

RESULTS AND DISCUSSION

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. Sometimes this is facilitated by a choice of substrate that is more reactive with the metal substituted enzyme than with the wild-type. In this

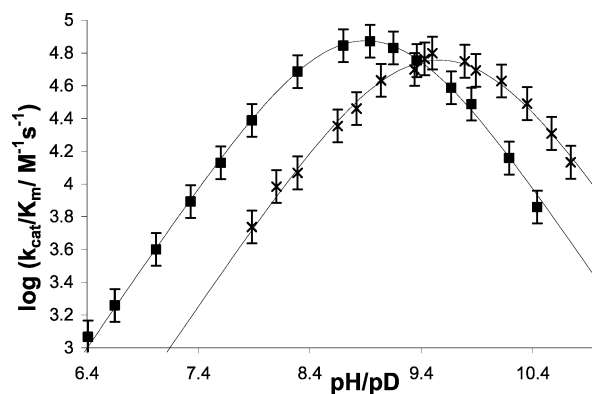
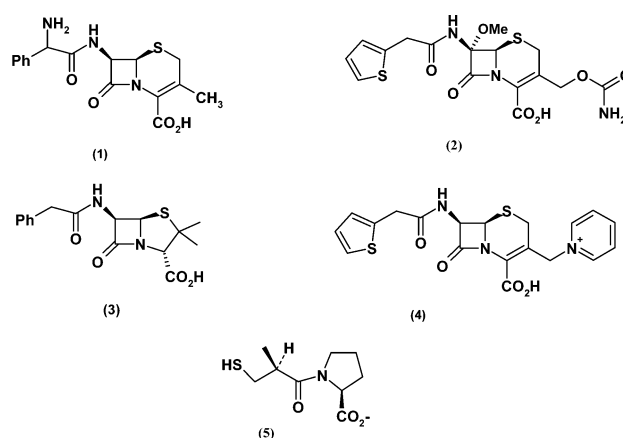
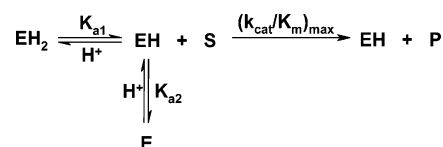


FIGURE 1: Plot of $\log(k_{\text{cat}}/K_{\text{m}})$ against pH(■)/pD(×) for CdBCII ([apoBcII] = 8×10^{-7} to 1.6×10^{-6} M, $[\text{Cd}^{2+}] = 1 \times 10^{-4}$ M) catalyzed hydrolysis of cefoxitin (1×10^{-4} M) in metal free buffer, 0.025 M, $[I] = 0.25$ M, at 30 °C; the solid lines are the calculated values using eq 3 and the parameters in Table 2.

Chart 1



Scheme 2



study, a background hydrolysis rate of all substrates (cephalexin (1), cefoxitin (2), benzylpenicillin (3), and cephaloridine (4) (Chart 1)) in the presence of the apo-enzyme was always determined and was insignificant, unless otherwise stated.

Cd Substituted BcII. The pH dependence of the second-order rate constant, $k_{\text{cat}}/K_{\text{m}}$, for the CdBcII catalyzed hydrolysis of cephalixin (1) and cefoxitin (2) is in both cases bell-shaped with a maximum value of $k_{\text{cat}}/K_{\text{m}}$ around pH = 8.8 (Figure 1). The maximum value of $k_{\text{cat}}/K_{\text{m}}$ for the hydrolysis of cephalixin is about 10-fold greater than that catalyzed by the zinc enzyme at pH = 7.5–8.5. It is therefore clear that the measured rate values represent the activity of the Cd enzyme. There is a first-order decrease in $k_{\text{cat}}/K_{\text{m}}$ on the acidic limb with increasing H^+ concentration and on the alkaline limb with increasing HO^- concentration (Figure 1). This variation of rate with pH can be explained by the ionization of two groups on the enzyme, one of which is required to be in its deprotonated form and the other in its protonated form for full catalytic activity (Scheme 2), with only the monoprotonated form, EH, having significant

Table 2: Calculated Values of the Rate and Acidity Constants from Scheme 2, for CdBCII ([apoBCII] = 1×10^{-7} to 1.6×10^{-6} M, $[\text{Cd}^{2+}] = 1 \times 10^{-4}$ M) Catalyzed Hydrolysis of Cephalixin and Cefoxitin (1×10^{-4} M) in Metal Free Buffer, 0.025 M, $[\text{I}] = 0.25$ M, at 30 °C

solvent	$(k_{\text{cat}}/K_{\text{m}})_{\text{max}}$ ($\text{M}^{-1} \text{s}^{-1}$)		pK_{a1}		pK_{a2}	
	cephalexin	cefoxitin	cephalexin	cefoxitin	cephalexin	cefoxitin
H ₂ O	$(2.69 \pm 0.3) \times 10^5$	$(2.00 \pm 0.2) \times 10^5$	8.69 ± 0.1	8.70 ± 0.1	9.41 ± 0.1	9.10 ± 0.1
D ₂ O	$(1.62 \pm 0.2) \times 10^5$	$(1.23 \pm 0.1) \times 10^5$	9.31 ± 0.1	9.23 ± 0.1	10.06 ± 0.1	9.89 ± 0.1
$(\Delta\text{pK}_{\text{a}}) \text{H}_2\text{O}/\text{D}_2\text{O}$	1.66 ± 0.1	1.62 ± 0.1	0.62 ± 0.1	0.53 ± 0.1	0.65 ± 0.1	0.79 ± 0.1

activity. Fitting the experimental data to eq 3, which describes Scheme 2, the pK_{a} values of the ionizing groups were found to be 8.69 ± 0.1 and 9.41 ± 0.1 for cephalixin and 8.70 ± 0.1 and 9.10 ± 0.1 for cefoxitin, respectively (Table 2). A similar pH–rate profile is seen in D₂O (Figure 1), and the kinetic solvent isotope effect, $k_{\text{H}_2\text{O}}/k_{\text{D}_2\text{O}}$, for $k_{\text{cat}}/K_{\text{m}}$ for the catalytically active species is 1.64.

$$\frac{k_{\text{cat}}}{K_{\text{m}}} = \frac{\left(\frac{k_{\text{cat}}}{K_{\text{m}}}\right)_{\text{max}}}{1 + \frac{[\text{H}^+]}{K_{\text{a1}}} + \frac{K_{\text{a2}}}{[\text{H}^+]} + \frac{K_{\text{a2}}}{K_{\text{a1}}}} \quad (3)$$

Varying the Cd^{2+} concentration from 1×10^{-5} M to 1×10^{-3} M, over the pH region studied, does not change the activity of the cadmium enzyme, suggesting that the activity of only one metalloenzyme species has been monitored, probably the dinuclear species, since the cadmium concentrations used are above the dissociation constant of the second cadmium ion (although a reported dissociation constant is 5.9×10^{-6} M (33), we have found, using ITC, that the dissociation constant for the second cadmium ion from the dinuclear CdBCII is in the submicromolar range in the pH region 6.5–8.5). The values of the first pK_{a} in H₂O and D₂O are identical, within error (± 0.1), for the CdBCII catalyzed hydrolysis of both substrates, which indicates that the ionization is substrate independent and probably due to an enzyme residue involved in catalysis or recognition. The $\text{pK}_{\text{a1}} = 8.70$ could be attributed to the Cd^{2+} -bound water, or to another enzyme residue which is required in its deprotonated form for enzyme activity. The difference in the ionization constants in D₂O and H₂O ($\Delta\text{pK}_{\text{a1}} = 0.53$ – 0.62) is compatible with the ionizable group being a weak acid, though not a thiol (44).

Benzylpenicillin (3) is a slightly poorer substrate for the Cd enzyme compared with ZnBCII: $k_{\text{cat}}/K_{\text{m}}$ at its pH maximum is about one-third of that observed for ZnBCII. After about 90% hydrolysis of benzylpenicillin, a second slower reaction is observed which, as shown by the addition of Zn^{2+} and varying the Cd concentration, is probably due to the hetero dinuclear enzyme, EZnCd. The second-order rate constant, $k_{\text{cat}}/K_{\text{m}}$, for the CdBCII catalyzed hydrolysis of benzylpenicillin was calculated from the first 90% of the progress curve, which gave a good fit to a first-order rate equation. Despite the difficulties due to the interference from ECdZn, there is a similar sigmoidal dependence in the pH–rate profile of CdBCII catalyzed hydrolysis of benzylpenicillin (not shown). The pK_{a} of the group required in its deprotonated form for activity, pK_{a1} , has a value of 8.3, slightly less than the corresponding value from the hydrolysis of cefoxitin and cephalixin (8.70), but the difference may be due to the

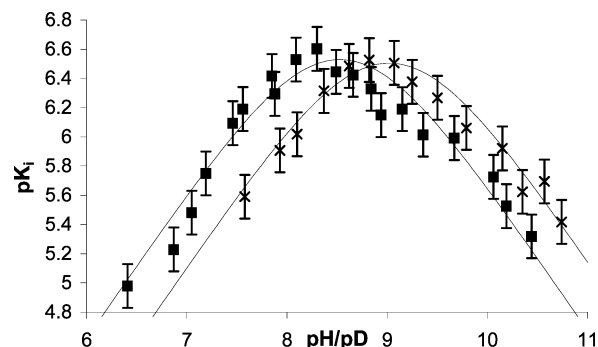


FIGURE 2: Plot of pK_{i} against $\text{pH}(\blacksquare)/\text{pD}(\times)$ for L-captopril (1×10^{-6} to 1×10^{-5} M) inhibition of CdBCII ([apoBCII] = 1×10^{-7} to 2×10^{-7} M, $[\text{Cd}^{2+}] = 1 \times 10^{-4}$ M) catalyzed hydrolysis of cephalixin (1×10^{-4} M) in metal free buffer, 0.025 M, $[\text{I}] = 0.25$ M, at 30 °C; the solid lines are the calculated values using eq 4 and the parameters in Table 3.

experimental limitations in measuring the activity of the holocadmium species with benzylpenicillin.

It has been suggested that competitive inhibitors of metallo- β -lactamases that contain a thiol group bind to the metalloenzyme by replacing the metal-bound water/hydroxide with the thiol group (45). Thus, the pH dependence of inhibition by a thiol may help to distinguish between the ionization of the metal-bound water and the ionization of another enzyme residue which is important for catalysis (39). L-Captopril (5) (Chart 1), a well-known angiotensin converting enzyme-blocking agent (46), was chosen for the inhibition studies because previous reports of its interaction with CdBCII are available (47), including EXAFS data which support the hypothesis that the thiol replaces the metal-bound water. L-Captopril shows a competitive inhibition constant, K_{i} , of 4.2×10^{-5} M with ZnBCII (39) and 1.5×10^{-6} M with CdBCII at $\text{pH} = 7.5$ (47). The inhibitor has two ionizable groups, a carboxylic acid and a thiol, with pK_{a} values of 3.7 and 9.7, respectively (48).

The pK_{i} –pH profile for L-captopril inhibition of CdBCII catalyzed hydrolysis of cephalixin is bell-shaped (Figure 2), with a maximum inhibition (lowest inhibition constant) around $\text{pH} = 8$. There is a first-order decrease in pK_{i} with increasing H^+ concentration on the acidic limb and a first-order decrease with increasing HO^- concentration on the basic side. Possible processes that could accommodate the experimental data are shown in Scheme 3, which assumes that the thiolate group of the inhibitor replaces the metal-bound water/hydroxide. When both the thiol and the metal-bound water are in their protonated states (equilibrium 1, Scheme 3), the concentration of the inhibited complex, ECdSR, decreases with pH. This could explain the decrease in pK_{i} on the acidic limb, because the thiol would be undissociated below pH 8. If one of these species is protonated and the other deprotonated, the binding is pH independent, equilibria 2 and 3. If both species are depro-

Scheme 3

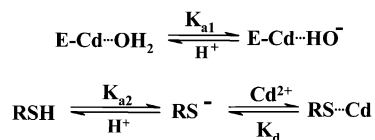
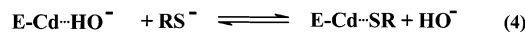
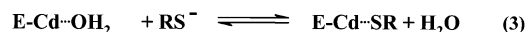
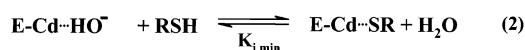
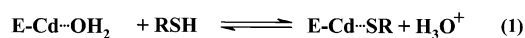


Table 3: Inhibition, Acidity, and Dissociation Constants for L-Captopril (1×10^{-6} to 1×10^{-5} M) Inhibition of CdBCII ([apoBCII] = 1×10^{-7} to 2×10^{-7} M, $[\text{Cd}^{2+}] = 1 \times 10^{-4}$ M) Catalyzed Hydrolysis of Cephalexin (1×10^{-4} M) in Metal Free Buffer, 0.025 M, [I] = 0.25 M, at 30 °C

solvent	$\text{p}K_{i\max}$	$\text{p}K_{a1}$	$\text{p}K_{a2}$	K_d (M)
H ₂ O	(7.29 ± 0.1)	(8.70 ± 0.1)	(9.80 ± 0.1)	$(3.72 \pm 0.4) \times 10^{-6}$
D ₂ O	(7.31 ± 0.1)	(9.25 ± 0.1)	(10.14 ± 0.1)	$(4.69 \pm 0.5) \times 10^{-6}$
H ₂ O/D ₂ O ($\Delta\text{p}K_a$)		0.55 ± 0.05	0.34 ± 0.04	

tonated, inhibition decreases with increasing HO^- concentration, equilibrium 4, which could explain the decrease in $\text{p}K_i$ on the alkaline limb.

According to Scheme 3, the variation of the inhibition constant, K_i , with pH is given by eq 4, where K_{a1} and K_{a2} correspond to the ionizations of the metal-bound water and of the thiol group in L-captopril, respectively, and K_d is the dissociation constant of the complex between cadmium ion and L-captopril. The experimental data were fitted to eq 4, and the resulting dissociation constants are given in Table 3.

$$K_i = K_{i(\min)} \left(1 + \frac{[\text{H}^+]}{K_{a1}} \right) \left(1 + \frac{K_{a2}}{[\text{H}^+]} \left(1 + \frac{[\text{Cd}^{2+}]}{K_d} \right) \right) \quad (4)$$

The values obtained for $\text{p}K_{a1}$, in H₂O and D₂O, from the inhibition studies, are very similar to those found from the hydrolysis experiments with cefoxitin and cephalexin, i.e., 8.70 ± 0.1 and 9.27 ± 0.1 , in H₂O and D₂O, respectively. The $\text{p}K_{a2}$ value (9.80) corresponds to the $\text{p}K_a$ of the thiol group in captopril (48), and the difference between the $\text{p}K_a$ in H₂O and D₂O, $\Delta\text{p}K_{a2} = 0.34$, is in agreement with the ionizable group being a thiol (44). The value of K_d (3.72×10^{-6} M) is in approximate agreement with the literature value (6×10^{-7} M) (48), considering the different conditions used (buffer, ionic strength). These values indicate that the model illustrated in Scheme 3 is valid and therefore that $\text{p}K_{a1}$ of 8.70 corresponds to the deprotonation of the Cd^{2+} -bound water.

The difference between the $\text{p}K_a = 5.60$ of the zinc-bound H₂O (39) and the $\text{p}K_a = 8.70$ of the Cd^{2+} -bound water in BcII, i.e., approximately 3 pH units, is greater than the difference of only 1 pH unit between the $\text{p}K_a = 8.96$ of the Zn aqua complex and the $\text{p}K_a = 10.08$ of the Cd aqua complex (49). The difference may be attributed to the smaller ionic radius of 0.74 Å for Zn^{2+} compared with 0.97 Å for

Cd^{2+} (50), which may lead to a higher coordination number for cadmium compared with zinc (50). For BcII, it has been shown by EXAFS (47) that Cd^{2+} is pentacoordinated in the His₃ site with two additional oxygen atoms, which were assumed to be two water molecules, whereas Zn^{2+} is tetraordinated in the His₃ site with only one water molecule/hydroxide ion (23). This tendency for a higher coordination number for cadmium presumably reduces the effective positive charge on the metal, which, in turn, leads to a higher $\text{p}K_a$ for the coordinated water. Furthermore, the larger ionic radius for Cd^{2+} makes it “softer” and a better coordinator to sulfur ligands compared with Zn^{2+} . The cysteine in the DCH site of BcII may have a greater effect on reducing the effective positive charge on Cd^{2+} , which would increase the $\text{p}K_a$ of the cadmium-bound water compared with the zinc-bound water. This could occur either directly, through metal ion coordination, or indirectly, through a cadmium coordinated water molecule hydrogen bonded to the nucleophilic hydroxide (51). Based on theoretical calculations, the presence of a complex hydrogen-bonding network in the active site of BcII has a role in lowering the $\text{p}K_a$ of the Zn^{2+} -bound water (52). This is experimentally supported by the low value of the $\text{p}K_a$ of the Zn^{2+} -bound water in BcII, which is lower than in other Zn hydrolytic enzymes: 6.8 in carbonic anhydrase (53) and 6.2 in carboxypeptidase A (54). It is possible that the distortion of the metal coordination sphere and the perturbation of this hydrogen-bonding network in the enzyme active site make a contribution to the higher $\text{p}K_a$ of the cadmium-bound water.

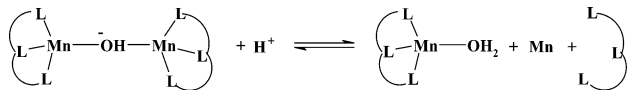
Other dinuclear metalloenzymes also show a significant increase in the $\text{p}K_a$ of the metal bridging water on replacing zinc by cadmium (53, 55, 56). The substitution of Cd^{2+} for the native Zn^{2+} in carbonic anhydrase shifts the $\text{p}K_a$ of the metal-bound water from 6.8 to 9.3, for esterase activity (53). In the phosphotriesterase found in soil bacteria, which catalyzes the hydrolysis of a wide variety of organophosphorus triesters (57), the cadmium-bound water has a $\text{p}K_a$ of 8.1, which is 2.3 pH units higher than that of the zinc-bound water (55). For the cadmium substituted horse liver alcohol dehydrogenase, PAC spectroscopy studies have suggested that the cadmium-bound water has a $\text{p}K_a$ of 11, which is about 2 pH units higher than the $\text{p}K_a$ of the zinc-bound water (56).

Mn Substituted BcII. It is of interest to determine the pH dependence of the catalytic efficiency of MnBcII with different β -lactam antibiotics and to compare it with the other metal substituted BcII species. A previous study of manganese substituted BcII catalyzed hydrolysis of benzylpenicillin reported a catalytic efficiency of about 8% of that of the zinc enzyme (43). The progress curves of MnBcII are markedly biphasic at low enzyme concentrations, the size of the “burst” being 10^4 times the concentration of the enzyme (58). However, at higher enzyme concentrations (5×10^{-7} to 2×10^{-6} M), the progress curves are in fact monophasic and lead to complete hydrolysis of the substrate. The substrates used in our study were cefoxitin (2), cephalexin (1), and benzylpenicillin (3). The second-order rate constants for MnBcII catalyzed hydrolysis have a bell-shaped pH profile (not shown) with a maximum value of k_{cat}/K_m , at pH 8.5–9.0, of 9.12×10^4 , 2.04×10^5 , and 2.88×10^4 M⁻¹ s⁻¹ for cefoxitin, cephalexin, and benzylpenicillin, respectively.

Table 4: Calculated Rate and Acidity Constants from Scheme 2, for MnBCII ([apoBCII] = 5×10^{-7} to 2×10^{-6} M, $[\text{Mn}^{2+}] = 10^{-4}$ – 10^{-3} M) Catalyzed Hydrolysis of Benzylpenicillin (1×10^{-3} M), Cefoxitin (1×10^{-4} M), and Cephalexin (1×10^{-4} M) in Metal Free Buffer, 0.025 M, $[\text{I}] = 0.25$ M, at 30 °C

	cefoxitin		cephalexin	benzylpenicillin
$[\text{Mn}^{2+}]$ (M)	10^{-4}	10^{-3}	10^{-3}	10^{-3}
$\text{p}K_{\text{a}1}$	8.65 ± 0.1	8.45 ± 0.1	8.39 ± 0.1	8.63 ± 0.1
$\text{p}K_{\text{a}2}$		9.0 ± 0.1	8.9 ± 0.2	9.21 ± 0.3
$(k_{\text{cat}}/K_{\text{m}})_{\text{max}}$ ($\text{M}^{-1} \text{s}^{-1}$)	$(1.23 \pm 0.2) \times 10^4$	$(9.12 \pm 1.0) \times 10^4$	$(2.04 \pm 0.2) \times 10^5$	$(2.88 \pm 0.4) \times 10^4$

Scheme 4



The experimental data were fitted to the mechanism proposed for CdBCII (Scheme 2, eq 3). A $\text{p}K_{\text{a}}$ value of about 8.5 was found for the ionizing group on the acidic limb ($\text{p}K_{\text{a}1}$) for the three substrates studied (Table 4). Due to the insufficient number of experimental data points on the alkaline limb, the $\text{p}K_{\text{a}}$ of the ionizing group required in its protonated form for activity ($\text{p}K_{\text{a}2}$) could not be accurately determined. There is a large dependence of enzyme activity on the concentration of metal ion. On the acid limb of the pH–rate profile there is an approximately first-order dependence on metal ion concentration which decreases with increasing pH. The maximum value of $k_{\text{cat}}/K_{\text{m}}$ also appears to shift to a higher pH at lower concentrations of Mn^{2+} .

Several models were considered in order to explain the metal ion and pH dependence of MnBCII catalyzed hydrolysis. The most acceptable of them involves an active dinuclear enzyme for which dissociation of the *second* manganese ion leads to enzyme inactivation (Scheme 4). Protonation of the metal bridging hydroxide ion results in the loss of a manganese ion and enzyme activity, which is in agreement with both the observed pH and metal ion dependence of the enzyme catalytic activity. It also implies that the binding of the second manganese ion is weak ($K_{\text{di}} > 1 \times 10^{-3}$ M), which is not unreasonable considering the hard acid character of the manganese ion which does not favor interactions with the soft (Cys221) and borderline (His263) bases present in the second binding site of BCII. The mononuclear MnBCII enzyme is not considered to be responsible for the observed catalytic activity because the dissociation constant of manganese from the active enzyme species (1×10^{-3} M) is much higher (10^6 – 10^4 -fold) than the dissociation constants of the other metal ions (zinc, cadmium, and cobalt) from the corresponding mononuclear BCII enzymes (Table 1).

The $\text{p}K_{\text{a}}$ of 8.5 thus appears to be due to the manganese bridging solvent molecule, which upon ionization can bind a second metal ion. Previous studies of dinuclear manganese enzymes and manganese substituted dizinc enzymes report $\text{p}K_{\text{a}}$ values between 7.0 and 8.0 for the manganese bridging water molecule (55, 59, 60).

Co Substituted BCII. For the substrates studied, namely, cefoxitin (2), cephaloridine (4), cephalexin (1), 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 measure K_{m} and $k_{\text{cat}}/K_{\text{m}}$, and this is especially true at lower pHs where K_{m} decreases with decreasing pH. In the following experiments two cobalt

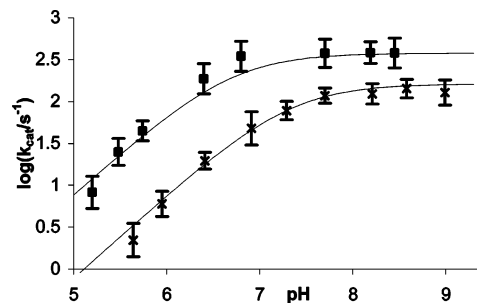


FIGURE 3: Plot of $\log k_{\text{cat}}$ against pH for CoBCII catalyzed hydrolysis of cefoxitin in 0.025 M buffer, $[\text{I}] = 0.25$ M, in the presence of 10^{-4} M Co^{2+} (x) and 10^{-3} M Co^{2+} (■); the solid lines are the calculated values using the parameters in Table 5.

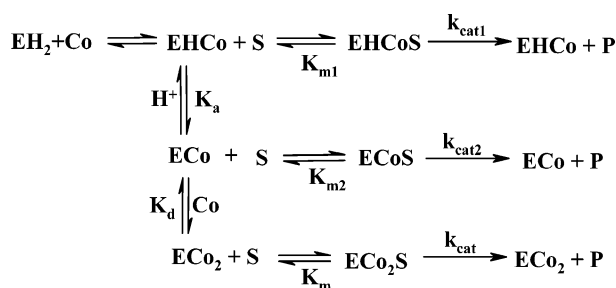
concentrations were used, 10^{-3} M and 10^{-4} M, which ensures that the monocobalt species is always present and that a variable dicobalt species could be present as the dissociation constants for loss of the metal ion from the mono- (K_{mono}) and dicobalt (K_{di}) species are 9×10^{-8} M and 6×10^{-5} M, respectively (33).

The catalytic constant, k_{cat} , for CoBCII catalyzed hydrolysis of cefoxitin (2) varies with pH and cobalt ion concentration as shown in Figure 3, which is indicative of a typical ionization controlling enzyme activity. Between pH 8 and 9 the reaction rate is pH independent, but decreases at lower pHs where 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 for a 10-fold decrease in cobalt ion concentration, k_{cat} decreases by approximately 10-fold, whereas in the pH–rate independent region it changes less than 3-fold. The kinetically important ionization in the k_{cat} –pH profile has an apparent $\text{p}K_{\text{a}}$ value of 7 and 6.5 for CoBCII catalyzed hydrolysis of cefoxitin at 10^{-4} and 10^{-3} M CoCl_2 , respectively (Figure 3).

The values of the second-order rate constant, $k_{\text{cat}}/K_{\text{m}}$, for Co–BCII catalyzed hydrolysis of cefoxitin are subject to considerable error but show an apparent bell-shaped 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, $k_{\text{cat}}/K_{\text{m}}$ increases by less than 2-fold for a 10-fold increase in metal ion concentration. The apparent $\text{p}K_{\text{a}}$ values for the acidic ionization in the pH– $k_{\text{cat}}/K_{\text{m}}$ profile are 6.3 and 5.8 for 10^{-4} and 10^{-3} M CoCl_2 , respectively. A similar trend for the variation of k_{cat} and $k_{\text{cat}}/K_{\text{m}}$ with pH at different CoCl_2 concentrations was observed using cephaloridine (4) and cephalexin (1) as a substrate (data not shown).

For the CoBCII catalyzed hydrolysis of benzylpenicillin (3) only the k_{cat} –pH profile was determined, due to the very low K_{m} values ($<10^{-4}$ M). At lower values of pH, k_{cat}

Scheme 5



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. Also, similar to cefoxitin, 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 (data not shown).

The variation of k_{cat} and $k_{\text{cat}}/K_{\text{m}}$ with pH and cobalt ion concentration is similar for all the 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 the 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} with decreasing pH and metal ion concentration suggests that there are at least two enzyme species, one of which has a greater value of k_{cat} 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 value of k_{cat} , and may effectively be inactive.

Several possible explanations for the metal ion concentration and pH dependencies have been considered, and that which is most compatible with all the data assumes that the monocobalt protonated (EHC_Co), the dicobalt (EC_Co_2) and the monocobalt deprotonated (EC_Co) enzyme species bind the substrate and are potentially catalytically active (Scheme 5). It was found that only the dinuclear enzyme has significant catalytic activity and that the residue required in deprotonated form for metal binding and catalysis has a pK_{a} of 6.52 ± 0.1 in the free enzyme (Table 5) (61). Pre steady state kinetics with CoBCII at low temperatures and with ZnBCII at low zinc ion concentrations support the hypothesis that only the dinuclear enzyme is responsible for catalytic activity but it becomes inactive following the loss of one metal ion during turnover (61). Our findings contradict the previous reports which suggest that the mononuclear BcII has activity comparable to that of the dinuclear species and is the one relevant for physiological activity (34).

Comparison between the Catalytic Properties of the Native Zinc and Metal Substituted BcII Enzyme. The catalytic activity of ZnBcII with cephalaxin (**1**) shows a bell-shaped pH-rate profile, Figure 4, as previously seen with other substrates (39), but with two plateau regions, one with a maximum $k_{\text{cat}}/K_{\text{m}}$ of about $5.9 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ between pH 5 and 6, and the other with a maximum $k_{\text{cat}}/K_{\text{m}}$ of about $2.4 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ between pH 7.5 and 8.5. The two maxima

indicate the existence of two active forms of the enzyme or of the substrate which react with different rates. As this behavior is only characteristic of cephalaxin, it is likely that the ionization, with an apparent pK_{a} of about 7, corresponds to the substrate (Scheme 6), probably to the amino group on the acylamido side chain, which has a reported pK_{a} value of 7.02 (62).

The experimental data were fitted to eq 5, which describes the model in Scheme 6, and the resulting parameters are given in Table 6. According to this analysis, cephalaxin with a protonated amino side chain is 4-fold less reactive than with the free base amine.

$$\frac{k_{\text{cat}}}{K_{\text{m}}} = \frac{\left(\frac{k_{\text{cat}}}{K_{\text{m}}}\right)_{\text{SH}} + \left(\frac{k_{\text{cat}}}{K_{\text{m}}}\right)_{\text{S}}}{1 + \frac{K_{\text{a}}}{[\text{H}^+]} + \frac{[\text{H}^+]}{K_{\text{a}}}} \quad (5)$$

A comparison of the pH- $k_{\text{cat}}/K_{\text{m}}$ profiles for cobalt, cadmium, and manganese substituted BcII species with that of the native zinc enzyme, for the hydrolysis of cephalaxin, is given in Figure 4 (Table 6). The different metallo substituted enzymes all show a bell-shaped pH-rate profile, with an ionization on the acidic limb (pK_{a1}) and an ionization on the alkaline limb (pK_{a2}) as described earlier for CdBCII (Scheme 2). All substituted metallo- β -lactamases have a higher maximum activity at their pH optimum than that of the native zinc species, but only CoBcII is more active than ZnBcII at pH 7. This emphasizes the importance of not comparing the activity of various metal substituted enzymes at a single pH if meaningful conclusions are to be drawn. The cobalt enzyme is remarkably about 100-fold more active than the native ZnBcII , and the cadmium and the manganese enzymes show a 10-fold greater activity, albeit at a higher pH.

For Cd and MnBcII , the activity was measured at pHs higher than 7 and there is no significant evidence of the ionization of the substrate having an effect on the kinetics, so the measured activity probably corresponds to the free amino species of cephalaxin. However, for CoBcII , the ionization seen in the pH- $k_{\text{cat}}/K_{\text{m}}$ profile may have contributions from both the ionization of the substrate and that of the enzyme (although a separate estimation of the two ionization constants is difficult, due to the limited number of data points). Hence the pK_{a} of the enzyme residue involved in catalysis may be less than the calculated value of 6.86 obtained from the pH-rate profile. The decrease in activity on the acidic limb of the pH-log $k_{\text{cat}}/K_{\text{m}}$ profile (Figure 4) has a slope slightly greater than 1, which may imply that both ionizations are kinetically important.

For CdBcII , where the rate is independent of external metal ion concentration, the ionization with a pK_{a} of 8.69 corresponds most probably to the pK_{a} of the metal-bound water. The 3 pH unit difference between the pK_{a} of the zinc-bound water and that of the cadmium-bound water indicates that cadmium has a lower positive charge density than zinc, which implies that it is a weaker Lewis acid. However, the metal-bound hydroxide ion resulting from the deprotonation of

Table 5: The Rate and Dissociation Constants, Defined in Scheme 5, for CoBCII Catalyzed Hydrolysis of Cefoxitin, Cephaloridine, and Benzylpenicillin in Buffer, 0.025 M, [I] = 0.25 M, at 30 °C

substrate	K_a (10^{-7} M)	K_d (10^{-5} M)	K_m (10^{-5} M)	k_{cat} (s^{-1})	K_{m1} (10^{-6} M)	K_{m2} (10^{-5} M)
cefoxitin	2.9 ± 0.8	6.0	31 ± 1.0	16 ± 2.0	12 ± 1.0	10 ± 2.0
cephaloridine	2.9 ± 0.8	6.0	4.0 ± 1.0	67 ± 10	3.0 ± 0.3	2.5 ± 0.5
benzylpenicillin	2.9 ± 0.8	6.0	16 ± 2.0	390 ± 20	12 ± 2.0	50 ± 10

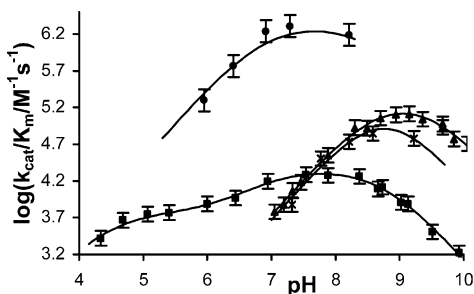
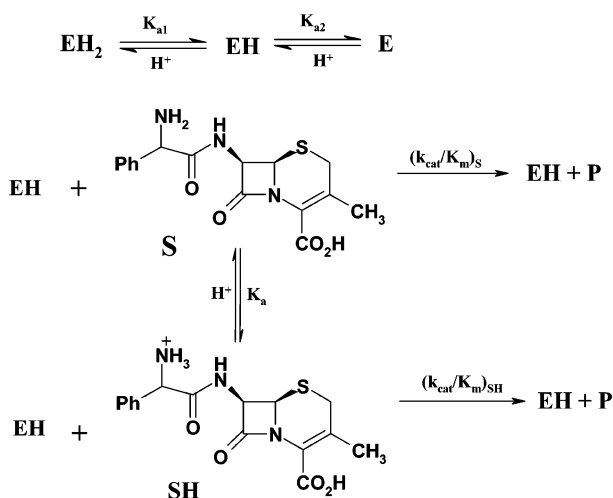


FIGURE 4: Plot of $\log k_{cat}/K_m$ for (■) ZnBCII ([apoBCII] = 1×10^{-7} to 4×10^{-7} M, $[Zn^{2+}] = 10^{-4}$ M), (●) CoBCII ([apoBCII] = 1×10^{-7} to 2×10^{-7} M, $[Co^{2+}] = 10^{-4}$ M), (▲) CdBCII ([apoBCII] = 1×10^{-7} to 2×10^{-7} M, $[Cd^{2+}] = 10^{-4}$ M), and (×) MnBCII ([apoBCII] = 5×10^{-7} to 1×10^{-6} M, $[Mn^{2+}] = 10^{-3}$ M) catalyzed hydrolysis of cephalixin (10^{-4} M) in buffer, 0.025 M, [I] = 0.25 M at 30 °C; the solid lines represent the calculated values using eq 5 (for ZnBCII) or eq 3 (for CoBCII, CdBCII, and MnBCII) and the parameters in Table 6.

Scheme 6



cadmium-bound water is a stronger nucleophile than the zinc-bound hydroxide. The fact that the cadmium enzyme is about 10-fold more reactive than the zinc enzyme suggests that the role of the metal ion is predominantly to provide the nucleophilic hydroxide, rather than to act as a Lewis acid to polarize the carbonyl group and stabilize the oxyanion tetrahedral intermediate. Also, if breakdown of the tetrahedral intermediate is rate-limiting (which is in agreement with the observed kinetic solvent isotope effects (39) and Table 2), a stronger Lewis acid would give a more stable oxyanion and so a system with less “electron-push” for the C–N bond fission. Hence, a too strong Lewis acid can impair catalysis by an effect both on the nucleophilicity of the metal-bound hydroxide and on the activation barrier for C–N bond fission.

Replacing Zn with Mn in BcII catalyzed hydrolysis of cephalixin also gives a 10-fold increase in the maximal activity, while the pK_a of the metal-bound water increases by approximately 3 pH units to 8.39. However, the maximal activity measured for MnBCII, even at the highest metal

concentrations used, is metal ion concentration dependent, so the real catalytic efficiency of the fully active MnBCII species is expected to be even higher. The implication is similar to that seen for Cd: a higher pK_a for the metal-bound water leads to a better catalyst, indicating a predominant role for nucleophilicity rather than Lewis acidity.

For the cobalt enzyme, the protonation event resulting in loss of activity at lower pHs may be due either to the metal-bound hydroxide ion or to an enzyme residue involved in metal binding. At maximum catalytic activity, the cobalt BcII enzyme is about 100-fold more efficient than the native ZnBCII, which is reflected predominantly by an increase in k_{cat} (24-fold) and a small decrease in K_m (4-fold). The 10-fold increase in the second-order rate constant, k_{cat}/K_m , for CoBCII compared with the manganese and the cadmium enzyme species is reflected in a large decrease in K_m (from more than 1×10^{-3} M for Cd and MnBCII to 7.8×10^{-5} M for CoBCII).

The relative reactivities of the various metal substituted BcII species depend on the substrate used to monitor activity. For example, with benzylpenicillin as substrate, the maximum second-order rate constants for the cadmium and manganese enzymes, k_{cat}/K_m , $2.63 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $2.88 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, are 3- and 30-fold, respectively, lower than the second-order rate constant for the ZnBCII enzyme ($8.7 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$) (Figure 5, Table 7). However, for both cadmium and manganese substituted enzymes, the real catalytic efficiency with benzylpenicillin is expected to be higher than the value found at 10^{-4} M $[Cd^{2+}]$ and 10^{-3} M $[Mn^{2+}]$, because the rate of hydrolysis is dependent on metal ion concentration at the pHs corresponding to maximum activity. For CoBCII, only the catalytic rate constant, k_{cat} , was determined with benzylpenicillin, and it is about 5-fold lower than that observed with the zinc enzyme.

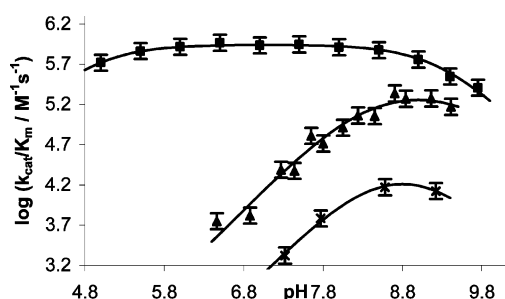
Cefoxitin (2) is a very poor substrate for the zinc enzyme ($k_{cat}/K_m = 100 \text{ M}^{-1} \text{ s}^{-1}$) (19), yet with the metal substituted β -lactamase, “normal” levels of catalytic activity are seen over the pH range 7.0–9.5 (Table 8, Figure 6). For other class B1 and class B3 metallo- β -lactamases, cefoxitin is a reasonably good substrate ($k_{cat}/K_m = 9.0 \times 10^4$ to $5.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$), but with modest k_{cat} values (1 – 10 s^{-1}). However cefoxitin inactivates the CphA enzyme from *A. hydrophila* (class B2 MBL) in a time dependent manner (19). The main difference between cefoxitin (2) and the other cephalosporins is the presence of a methoxy group in the 7- α position on the β -lactam ring. It appears, therefore, that the unusual low catalytic activity of ZnBCII toward cefoxitin, compared to other cephalosporins, is due to steric hindrance brought about by the methoxy substituent during the catalytic cycle, as previously proposed for serine β -lactamases (63). However, 7- α -methoxy substituted cephalosporins show similar reactivities toward alkaline hydrolysis to their unsubstituted counterparts (64). It is thus interesting that metal substitution in the active site of BcII can circumvent these negative steric

Table 6: Rate and Acidity Constants for the Metal Substituted BCII Catalyzed Hydrolysis of Cephalixin in Buffer, 0.025 M, [I] = 0.25 M at 30 °C, Concentration of Metal Ion = 10^{-4} M, Except $[\text{Mn}^{2+}] = 10^{-3}$ M

enzyme	$(k_{\text{cat}}/K_m)_s$ ($\text{M}^{-1} \text{s}^{-1}$)	$(k_{\text{cat}})_s$ (s^{-1})	$(K_m)_s$ (M)	$\text{p}K_{\text{a}1}$	$\text{p}K_{\text{a}2}$	$\text{p}K_{\text{a}}$
ZnBCII	$(2.40 \pm 0.3) \times 10^4$	7.30 ± 1.4	$(3.0 \pm 0.4) \times 10^{-4}$	4.40 ± 0.1	8.77 ± 0.1	6.94 ± 0.1
CoBCII	$(2.24 \pm 0.4) \times 10^6$	174 ± 2.1	$(7.8 \pm 1.0) \times 10^{-5}$	≤ 6.86	8.51 ± 0.1	
CdBCII	$(2.69 \pm 0.3) \times 10^5$	> 270	$> 10^{-3}$	8.69 ± 0.1	9.31 ± 0.1	
MnBCII	$(2.04 \pm 0.3) \times 10^5$	> 204	$> 10^{-3}$	8.39 ± 0.1	8.9 ± 0.2	

Table 7: Rate and Acidity Constants for the Metal Substituted BCII Catalyzed Hydrolysis of Benzylpenicillin in Buffer, 0.025 M, [I] = 0.25 M at 30 °C, Concentration of Metal Ion = 10^{-4} M, Except $[\text{Mn}^{2+}] = 10^{-3}$ M

enzyme	k_{cat}/K_m ($\text{M}^{-1} \text{s}^{-1}$)	k_{cat} (s^{-1})	K_m (M)	$\text{p}K_{\text{a}1}$	$\text{p}K_{\text{a}2}$
ZnBCII	$(8.7 \pm 1.0) \times 10^5$	950 ± 120	$(1.1 \pm 0.2) \times 10^{-3}$	4.80 ± 0.1	9.30 ± 0.1
CoBCII		200 ± 25			
CdBCII	$(2.63 \pm 0.2) \times 10^5$	> 1200	$> 3.0 \times 10^{-3}$	8.32 ± 0.1	9.68 ± 0.1
MnBCII	$(2.88 \pm 0.4) \times 10^4$	> 90	$> 3.0 \times 10^{-3}$	8.63 ± 0.1	9.21 ± 0.3

FIGURE 5: Plot of $\log k_{\text{cat}}/K_m$ for (■) ZnBCII ([apoBCII] = 1×10^{-8} to 4×10^{-8} M, $[\text{Zn}^{2+}] = 10^{-4}$ M), (▲) CdBCII ([apoBCII] = 1×10^{-7} to 2×10^{-7} M, $[\text{Cd}^{2+}] = 10^{-4}$ M), and (×) MnBCII ([apoBCII] = 5×10^{-7} to 1×10^{-6} M, $[\text{Mn}^{2+}] = 10^{-3}$ M) catalyzed hydrolysis of benzylpenicillin (2.5×10^{-4} M) in buffer, 0.025 M, [I] = 0.25 M at 30 °C; the solid lines represent the calculated values using eq 3 and the parameters in Table 7.

effects, particularly considering the different Lewis acid character of the metal ion used (Mn^{2+} , hard; Co^{2+} , borderline; Zn^{2+} , borderline; Cd^{2+} , soft). It is also worth noting that the catalytic activities of the native and metal substituted enzyme species, both k_{cat} and k_{cat}/K_m , increase with increasing the $\text{p}K_{\text{a}}$ of the metal-bound water ($\text{Zn}^{2+} < \text{Co}^{2+} < \text{Mn}^{2+} < \text{Cd}^{2+}$), i.e., with increasing the nucleophilicity of the metal-bound hydroxide ion.

The $\text{p}K_{\text{a}1}$ of 6.3 observed with CoBcII and cefoxitin indicates that the $\text{p}K_{\text{a}1}$ of about 6.9 seen with cephalixin as substrate reflects the ionization of the ammonium ion side chain. The $\text{p}K_{\text{a}1}$ and $\text{p}K_{\text{a}2}$ values obtained with CoBcII and cefoxitin, 6.3 and 8.5, respectively, are also compatible with the pH–rate profile seen with cephalixin (Figure 4).

CONCLUSION

The observations regarding the relative catalytic efficiencies of the different metal substituted BcII species with different substrates suggest that BcII catalysis is tolerant of active-site metal ion substitution. One main advantage of zinc over the other metal ions studied is that it has higher affinity for the enzyme active site, so the concentrations required for full catalytic activity are lower, and closer to those physiologically available (65). Another reason why zinc is preferred is that, unlike cadmium and manganese, it is able to provide the fully ionized metal-bound water hydroxide nucleophile at neutral pH.

Compared with the zinc enzyme, the catalytic rate constants, k_{cat} , are, as a general trend, significantly greater

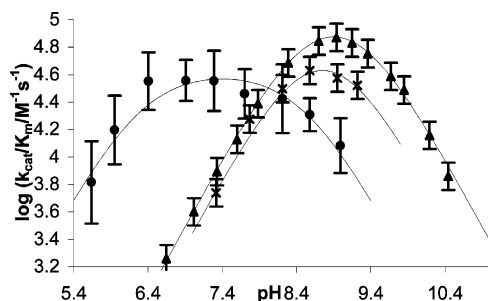
for the hydrolysis of the cephalosporins cephalixin and cefoxitin, catalyzed by CdBcII, and to a lesser extent the manganese enzyme, and are approaching the high k_{cat} levels seen with ZnBcII and penicillins (19). Also, the k_{cat} values for cephalixin and benzylpenicillin with CoBcII are very similar, while with ZnBcII the value of k_{cat} for benzylpenicillin is 100-fold greater than that for cephalixin hydrolysis. The k_{cat} values for the ZnBcII catalyzed hydrolysis of cephalosporins are 10–100 fold less than those for penicillins (19), which may be due either to a higher degree of stabilization of the Michaelis complex or to a less stable transition state. On replacing zinc with cadmium this effect is decreased (penicillin shows only 5–10-fold higher k_{cat} values than cephalosporins), which may be correlated with the increase in the $\text{p}K_{\text{a}}$ of the metal-bound water, and the formation of a better hydroxide nucleophile. The second-order rate constant for the hydroxide ion catalyzed hydrolysis of penicillins can be from 5-fold greater to 5-fold less than those for cephalosporins (66) depending on the nature of the substituent at C3.

The value of $\text{p}K_{\text{a}1}$ varies significantly with the nature of the metal ion: from 5.6 (39) for the zinc enzyme to 8.70 for the cadmium enzyme. It is also dependent on the concentration of the metal ion for zinc (39), cobalt, and manganese. These results indicate that $\text{p}K_{\text{a}1}$ corresponds to a group on the enzyme interacting closely with the metal ion, very likely a metal ligand. For the cadmium and manganese enzymes, kinetic evidence strongly suggests that the ionizing group of $\text{p}K_{\text{a}1}$ is the metal-bound water, which is required in deprotonated form for activity. For the zinc and cobalt enzymes, $\text{p}K_{\text{a}1}$ may correspond either to the metal-bound water or to a metal binding enzyme residue (such as the Cys221 or the Cys221-His263 pair (61)). The $\text{p}K_{\text{a}}$ of the Cys (Cys-His) pair in the apo-enzyme was found to be 7.85, but may decrease in the mononuclear species. Moreover, in the presence of external metal ion concentration, the apparent $\text{p}K_{\text{a}}$ value for this ionization ($\text{p}K_{\text{a}1}$) is expected to further decrease with increasing the affinity of the metal ion for the second binding site in the enzyme (Scheme 7) according to eq 6, which is in agreement with the experimental values obtained: $\text{p}K_{\text{a}1}^{\text{Zn}} = 4.6$ ($[\text{Zn}^{2+}] = 10^{-4}$ M, $K_d = 1.5 \times 10^{-6}$ M), $\text{p}K_{\text{a}1}^{\text{Co}} = 6.3$ ($[\text{Co}^{2+}] = 10^{-4}$ M, $K_d = 6 \times 10^{-5}$ M).

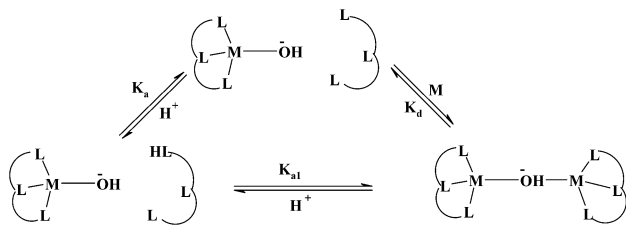
$$\text{p}K_{\text{a}1} = \text{p}K_{\text{a}} + \log \left(\frac{K_d}{[\text{M}^{2+}]} \right) \quad (6)$$

Table 8: Rate and Acidity Constants for the Metal Substituted BCII Catalyzed Hydrolysis of Cefoxitin in Buffer, 0.025 M, [I] = 0.25 M at 30 °C, Concentration of Metal Ion = 10^{-4} M, Except $[\text{Mn}^{2+}] = 10^{-3}$ M

enzyme	k_{cat}/K_m ($\text{M}^{-1} \text{s}^{-1}$)	k_{cat} (s^{-1})	K_m (M)	pK_{a1}	pK_{a2}
ZnBCII	100 ^a	0.2 ^a	$2.1 \times 10^{-3\text{a}}$		
CoBCII	$(4.3 \pm 0.5) \times 10^4$	4.7 ± 0.8	$(4.0 \pm 0.6) \times 10^{-5}$	6.3 ± 0.1	8.5 ± 0.1
CdBCII	$(2.0 \pm 1.0) \times 10^5$	>200	$>1.0 \times 10^{-3}$	8.7 ± 0.1	9.1 ± 0.1
MnBCII	$(9.1 \pm 0.2) \times 10^4$	>91	$>1.0 \times 10^{-3}$	8.5 ± 0.1	9.0 ± 0.1

^a From reference 19.FIGURE 6: Plot of $\log k_{\text{cat}}/K_m$ for (●) CoBCII ([apoBCII] = 5×10^{-7} to 2×10^{-6} M, $[\text{Co}^{2+}] = 10^{-4}$ M), (▲) CdBCII ([apoBCII] = 8×10^{-7} to 1.6×10^{-6} M, $[\text{Cd}^{2+}] = 10^{-4}$ M), and (×) MnBCII ([apoBCII] = 5×10^{-7} to 2×10^{-6} M, $[\text{Mn}^{2+}] = 10^{-3}$ M) catalyzed hydrolysis of cefoxitin (10^{-4} M) in buffer, 0.025 M, [I] = 0.25 M at 30 °C; the solid lines represent the calculated values using eq 3 and the parameters in Table 8.

Scheme 7



The previous studies by Bounaga (39) at lower pHs and zinc ion concentrations, where the zinc enzyme activity decreases with two zinc dependent protonation events, can be accommodated to a similar model: one protonation corresponds to a metal ligand enzyme residue and the other to the metal-bound water, but addition of zinc ion reverts both protonation events, which restores the enzyme activity. In the case of CoBCII, if pK_{a1} corresponds to a metal ligand enzyme residue, then, as the rate of hydrolysis is pH independent at higher pHs, the pK_{a} of the cobalt-bound water is expected to be lower than 6.3.

The variation of pK_{a1} with the nature and the concentration of the metal ion is consistent with a general theme where the protonation of the metal bridging hydroxide ion ($\text{M}-\text{OH}-\text{M}$) liberates one metal ion and gives the mononuclear inactive enzyme ($\text{M}-\text{OH}_2$) (61).

The value of pK_{a2} , corresponding to an enzyme residue required in its protonated form for activity, does not vary significantly with the nature of the substrate and the metal ion, and is typically in the pH region 8.5–9.5. An enzyme group of $\text{pK}_{\text{a}} = 9.3$ was found to be required in its protonated form for an optimum interaction with a mercaptocarboxylate inhibitor (61). It is thus possible that pK_{a2} corresponds to a carboxylate binding residue in the enzyme, possibly to the side chain of Lys 224 or to the apical water molecule coordinated to the second metal ion, which may be replaced upon substrate binding (67).

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