The mechanism of catalysis and the inhibition of the *Bacillus cereus* zinc-dependent β-lactamase

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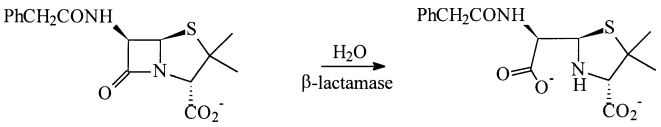
The plot of k_{cat}/K_m against pH for the *Bacillus cereus* 569/H β-lactamase class B catalysed hydrolysis of benzylpenicillin and cephalosporin indicates that there are three catalytically important groups, two of pK_a 5.6 ± 0.2 and one of pK_a 9.5 ± 0.2. Below pH 5 there is an inverse second-order dependence of reactivity upon hydrogen ion concentration, indicative of the requirement of two basic residues for catalysis. These are assigned to zinc(II)-bound water and Asp-90, both with a pK_a of 5.6 ± 0.2. A thiol, *N*-(2′-mercaptoethyl)-2-phenylacetamide, is an inhibitor of the class B enzyme with a K_i of 70 μM. The pH-dependence of K_i shows similar pH inflections to those observed in the catalysed hydrolysis of substrates. The pH-independence of K_i between pH 6 and 9 indicates that the pK_a of zinc(II)-bound water must be 5.6 and not the higher pK_a of 9.5. The kinetic solvent isotope effect on k_{cat}/K_m is 1.3 ± 0.5 and that on k_{cat} is 1.5. There is no effect on reactivity by either added zinc(II) or methanol. The possible mechanisms of action for the class B β-lactamase are discussed, and it is concluded that zinc(II) acts as a Lewis acid to stabilize the dianionic form of the tetrahedral intermediate and to provide a hydroxide-ion bound nucleophile, whereas the carboxylate anion of Asp-90 acts as a general base to form the dianion and also, presumably, as a general acid catalyst facilitating C–N bond fission.

INTRODUCTION

β-Lactamases are extracellular or periplasmic enzymes produced by bacteria which give the organisms resistance to the normally lethal action of β-lactam antibiotics by catalysing their hydrolysis (Scheme 1) [1–3]. The enzymes have been divided into four classes on the basis of their primary structures and catalytic mechanisms. Enzymes of classes A, C and D are active-site-serine enzymes but can be distinguished on the basis of their primary structures [4–7].



Scheme 1

Class B β-lactamases are metalloproteins which require zinc(II) ions for their activity. The first of these enzymes to be discovered was called β-lactamase II [8] and is produced by *Bacillus cereus*, which also produces two distinct class A enzymes. There are at least ten bacterial sources of the metallo-enzyme, including those found in *Pseudomonas maltophilia* L-1) [9–10], which also produces a class C enzyme, *Aeromonas hydrophila* (A2) [11] and *Bacteroides fragilis* [12,13].

Although these metallo-enzymes were initially thought to be clinically unimportant, some pernicious strains have been shown to owe their antibiotic resistance to their ability to produce zinc-containing β-lactamases [14]. The mechanism-based inactivators which have been utilized to fight the serine enzymes are generally ineffective against the Zn(II)-dependent enzymes, and, at present, there are no clinically useful inhibitors known. Metallo-β-lactamases are thought to be mechanistically different from those β-lactamases which have serine at the active site but little is known about the detailed mechanism of action of the zinc(II) enzymes. One of the main characteristics of the zinc enzyme is its ability to catalyse the hydrolysis of nearly all β-lactams, including carbapenems [3]. A review [15] of the catalytic properties of the well-characterized class B β-lactamases show that the *A. hydrophila* enzymes clearly exhibits the most specific substrate profile, whereas the other enzymes are rather broad-spectrum.

The sequence of the metallo-β-lactamases has been established and they all contain a single peptide chain composed of 220–230 residues [16–22]. A sequence comparison indicates that the *P. maltophilia* enzyme is only remotely related to the others, which do appear to constitute a more homogeneous group and exhibit 37 strictly conserved residues. Surprisingly, His-86, one of the histidine residues which seems to be involved as a Zn(II)-binding ligand in the *B. cereus* enzyme, is replaced by an asparagine residue in the *A. hydrophila* β-lactamase [16].

Of particular ambiguity is the number of zinc ions per molecule of β-lactamase. A low-resolution crystal structure of the *B. cereus* (569/H/9) β-lactamase showed 1 mol of Zn(II) in the active site bound by one cysteine and three histidine residues [23]. A second metal-binding site was identified but this bound Zn(II) only weakly. However, more recently, a second crystal structure of the same enzyme showed only a single metal-binding site, with other significant differences. The zinc ion, in the *B. cereus* (569/H/9) β-lactamase is co-ordinated by three histidine residues (86, 88 and 149) and, probably, a water molecule in a distorted tetrahedral arrangement [24]. Equilibrium dialysis [25] and ^{1}H-NMR [26] indicate that the *B. cereus* II enzyme is capable of binding two zinc ions but computer-assisted molecular modelling suggests that there is just one major metal-ion binding site [23]. As with many metallo-β-lactamases, the zinc ion of the *B. cereus* II enzyme can be replaced by different metal ions and still retain some β-lactamase activity [25]. Most mechanistic and structural information is derived from the *B. cereus* enzyme. However, a structure has very recently been reported for the binuclear zinc

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β-lactamase from *Bact. fragilis* [27,28], which tightly binds both Zn(II) ions although the loss of a single Zn(II) was not catastrophic for β-lactamase activity [29].

The mechanism of action of *B. cereus* II metallo-β-lactamase is generally thought to be similar to that of carboxypeptidase A and to involve a water molecule bound to the zinc ion of the active site, which attacks the carbonyl group of the β-lactam ring. It was originally proposed that Glu-37 acted as a general base, which deprotonated the water molecule with subsequent donation of the proton to the nitrogen atom of the β-lactam ring to cause cleavage [30]. However, Glu-37 is too far from the zinc ion to perform this function and site-directed mutagenesis studies have shown that this glutamate is not essential for the catalytic function of the enzyme [31]. It has been demonstrated that Asp-90 is essential for enzyme activity and it was consequently suggested that this residue acts as the general base to assist in the hydrolysis of the amide bond of β-lactam substrates [24,32].

The detailed function of the amino acids in the active site of the *B. cereus* II metallo-β-lactamase requires further elucidation, but, in summary, it is not known whether the hydrolysis of β-lactams catalysed by class B β-lactamase occurs by general base catalysis or whether zinc acts as an electrophile or as a provider of zinc-bound hydroxide ion. Furthermore, if there is a second binding site for zinc its exact role is not known. As a contribution to understanding the mechanism of zinc β-lactamase we report the pH dependence and kinetic solvent-isotope effects for the enzyme-catalysed hydrolysis of penicillins and cephalosporins. Based on analogy with zinc proteases, it is probable that the mechanism does at least involve formation of a tetrahedral intermediate which is stabilized by the zinc ion. Other ligands should be capable of replacing the oxygen anion of the tetrahedral intermediate and in the present study we also report the synthesis of analogues based on sulphur donors and their inhibition of the class B metallo-β-lactamase from *B. cereus* 569/H. The pH-dependence of the inhibition constants is used to make deductions about the pK_a of the zinc-bound water.

EXPERIMENTAL

Synthesis of *N*-(2′-mercaptoethyl)-2-phenylacetamide

Phenylacetylchloride (5.5 mmol) was added dropwise to a solution of cystamine (2.5 mmol) in water (40 ml) containing NaOH (11.25 mmol) and was cooled to 0 °C. The mixture was vigorously stirred. At the end of the reaction, the white precipitate formed was filtered and washed with water. The solid was recrystallized from hot methanol and subjected to TLC in chloroform/methanol/ammonia (10:12:1, by vol.). The disulphide obtained was reduced by adding sodium borohydride (88 mmol) to a solution of the disulphide (4.4 mmol) in ethanol/water (5:3, v/v). The mixture was heated to 90 °C and stirred for 1 h. The reaction was stopped by cooling the mixture, which was then acidified to pH 2 with dilute HCl. After evaporation of the ethanol, the product was separated by filtration and the filtrate was extracted with chloroform to obtain the remaining thiol. The combined extract was washed with water and dried over anhydrous Na_2SO_4. The solvent was removed by rotary evaporation to give a white product. The total yield was 87%. The product was separated by TLC in chloroform/methanol (99:1, v/v). After exposure of the TLC plates to iodine vapour, the thiol appeared as a white spot on a tanned background.

Some characteristics of the thiol were: IR (Nujol), ν_{max} 3253 (amide NH), 2544 (SH) and 1637 cm^{-1} (amide CO); ^{1}H-NMR, *J* values are given in Hz, δ_H (C^2HCl_3) 7.30 (5 H, m, Ar-H), 5.75 (1 H, NH), 3.55 (2 H, s, CH_2-Ar), 3.35 (2 H, q, *J* 6.6, H-1′), 2.52 (2 H, dt, *J* 6.6 and 8.5, H-2′) 1.18 (1 H, t, *J* 8.5, SH).

A pK_a of 9.5 ± 0.10 of the thiol was determined from the dependence of the absorbance at 238 nm as a function of pH.

Kinetic studies

pH Dependence of enzyme activity and solvent kinetic isotope effects

Kinetic studies were carried out using the class B β-lactamase enzyme from *B. cereus* 569/H. The buffers used were acetate (pH 4.0–5.3), Mes (pH 5.6–6.5), Mops (pH 7.0–8.0), Taps [N-Tris(hydroxymethyl)methyl-3-aminopropane] (pH 8.0–9.4), Capso [3-(cyclohexylamino)-2-hydroxysulphonic acid] (pH 9.1–10.0), hexafluoroisopropanol (pH 9.0–10.5) and Caps [3-(cyclohexylamino)-1-propanesulphonic acid] (pH 10.1–11) at 30 °C and 0.05 M, with the ionic strength maintained at 1.0 M with NaCl. The concentration of enzyme used was 0.04–2.0 μM, that of zinc ions was normally 10-fold greater than the enzyme concentration, that of cephaloridine 0.12–0.2 mM and benzylpenicillin 0.92–1.0 mM. Hydrolysis of the substrate was followed by measuring the decrease in absorbance at 260 and 230 nm for cephaloridine and benzylpenicillin respectively as a function of time. The kinetic parameters were determined as described previously [33] and curve fitting was achieved using ENZFITTER (Elsevier Biosoft, Cambridge, U.K.) or SCIENTIST (MicroMath Scientific Software, Salt Lake City, UT, U.S.A.) software.

Inhibition studies

Inhibition studies were carried out using the buffers used for the hydrolysis studies. The kinetic parameters were determined from the second-order rate constant k_{cat}/K_m. Inhibition constants were calculated using the equation (eqn. 1) for competitive inhibition:

$$\text{Rate} = \frac{k_{cat} \cdot [E] \cdot [S]}{[S] + K_m\left(\frac{K_i + [I]}{K_i}\right)} \quad (1)$$

RESULTS

Buffer and ionic-strength effects

It is relatively rare for enzyme kinetic studies to involve the effects of different buffers, buffer concentrations and changes in ionic strength. In most cases these effects are usually minor therefore the lack of control of these parameters is of little consequence. However, for those studies requiring the careful deduction of kinetic parameters, for example, solvent-isotope effects, pK_as from pH-rate profiles etc., buffer and ionic-strength control is important.

The kinetic constants were obtained from either initial rates or, more commonly, by following the entire course of the reaction. Below saturation, good pseudo-first-order rate constants, k_{obs}, were obtained, which were shown to be first-order in enzyme concentration and therefore, the second-order rate constant k_{cat}/K_m, was derived by dividing k_{obs} by the concentration of enzyme used.

The effect of ionic strength on the second-order rate constant k_{cat}/K_m for the *B. cereus* β-lactamase-II-catalysed hydrolysis of cephaloridine is shown in Figure 1. At a constant buffer concentration, a change in ionic strength from 0.01–1.0 M (NaCl) changed the k_{cat}/K_m about two-fold. The ionic strength effect

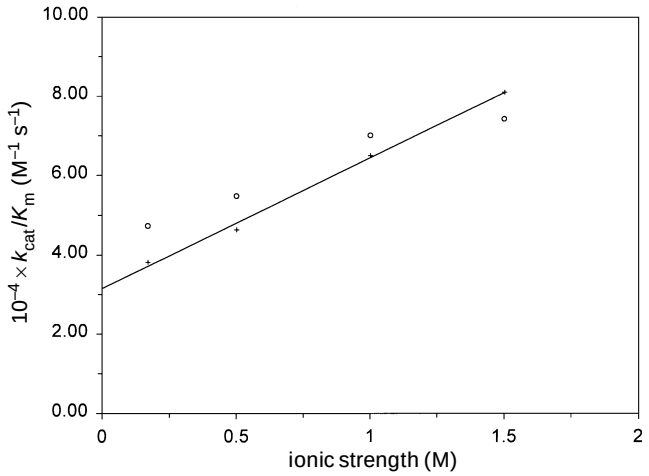


Figure 1 Dependence on ionic strength for the β-lactamase-catalysed hydrolysis of cephaloridine

The dependence of the second order rate constant k_{cat}/K_m for the β-lactamase II catalysed hydrolysis of cephaloridine upon the ionic strength of the medium (adjusted with NaCl) at 30 °C, pH 7.0, with Mops (○) or phosphate (+) at a constant buffer concentration. The ionic strength was calculated from $0.5 \Sigma C_i Z_i^2$ for all ionic species present, including the buffer.

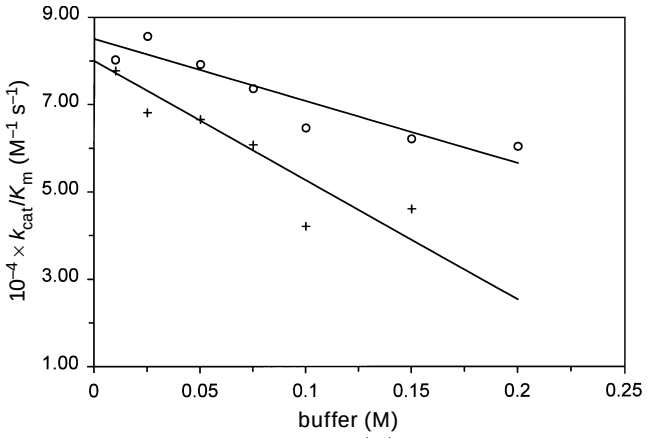


Figure 2 Dependence on buffer concentration for the β-lactamase-catalysed hydrolysis of cephaloridine

The dependence of the second-order rate constant k_{cat}/K_m for the β-lactamase II catalysed hydrolysis of cephaloridine upon buffer concentration (○, Mops; +, phosphate) at 30 °C, pH 7.0. The ionic strength was kept constant throughout at 1.0 M by varying the concentration of NaCl.

was slightly greater when phosphate buffer was compared with Mops.

Figure 2 shows the effect of varying the buffer concentration and buffer type on the rate of the enzyme-catalysed hydrolysis of cephaloridine in a solution maintained at a constant total ionic strength of 1.0 M, by adjusting the concentration of NaCl to compensate for changes in ionic strength brought about by changes in the buffer concentration. Both Mops and phosphate buffers decrease the rate of hydrolysis; 0.15 M phosphate causes a 50% reduction in k_{cat}/K_m compared with zero buffer concentration. The effect of Mops was slightly less, particularly at low buffer concentrations. Consequently, most studies were undertaken at 0.05 M Mops buffer.

Table 1 Effect of zinc(II) concentration on *B. cereus* β-lactamase class B activity

The effect of zinc(II) concentration on the *B. cereus* β-lactamase class B (1.0×10^{-7} M) catalysed hydrolysis of cephaloridine (1.52×10^{-4} M) in Mops buffer (0.05 M) at pH 7.01, maintained at constant ionic strength of 1.0 M (NaCl) at 30.0 °C.

Zn(II) concentration (M)	$10^{-4} \times k_{cat}/K_m$ ($M^{-1} \cdot s^{-1}$)
1.0×10^{-7}	7.54
2.0×10^{-7}	7.96
1.1×10^{-6}	7.39
1.0×10^{-5}	8.42
1.0×10^{-4}	9.19
1.0×10^{-3}	10.28

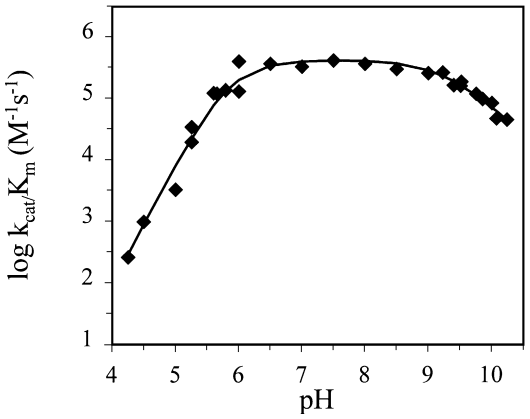


Figure 3 Dependence on pH for the β-lactamase-catalysed hydrolysis of benzylpenicillin

The pH dependence of log k_{cat}/K_m for β-lactamase-catalysed hydrolysis of benzylpenicillin at 30 °C and constant ionic strength of 1.0 M (NaCl). The solid line was calculated using eqn. (2) in the text.

Dependence of k_{cat}/K_m on Zn(II) concentration

Some of the metallo-β-lactamases appear to have only one binding site for zinc(II), whereas others clearly have two. The effect of zinc(II) concentration on the rate of hydrolysis of cephaloridine was examined at pH 7.0 in 0.05 M Mops buffer at a constant ionic strength of 1.0 M (NaCl). Table 1 shows the values of k_{cat}/K_m with zinc(II) concentrations varying from 1.0×10^{-7} to 1.0×10^{-3} M zinc sulphate. The second-order rate constant increased, but by only 36% over a 10^4-fold change in concentration of zinc(II). There was obviously no dramatic effect of any secondary binding site upon catalytic activity.

pH dependence of the rate of hydrolysis

Although the pH dependence on the rate of hydrolysis of β-lactams has been reported previously, the numbers of data points at acid pH were limited [34]. Figure 3 and Figure 4 show pH versus the log of the rate constant, k_{cat}/K_m, for the hydrolysis of benzylpenicillin and cephaloridine, respectively, catalysed by class B β-lactamase from *B. cereus*. Both showed characteristic bell-shaped curves, but, surprisingly, the slope of the acidic part of the curve was clearly 2.0 and not the usual 1.0. However, there appeared to be only one discernible inflection point. The variations in kinetic constants are the result of reversible ionization

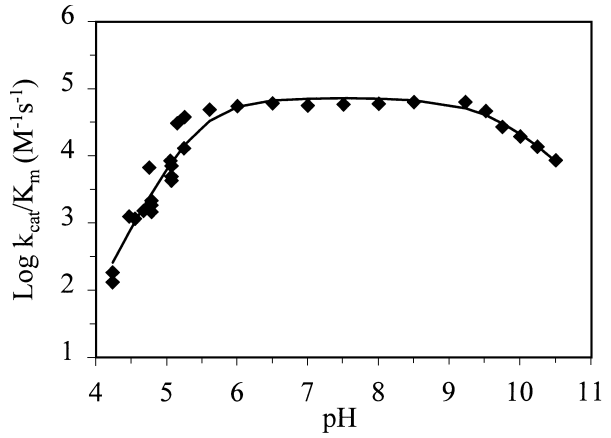
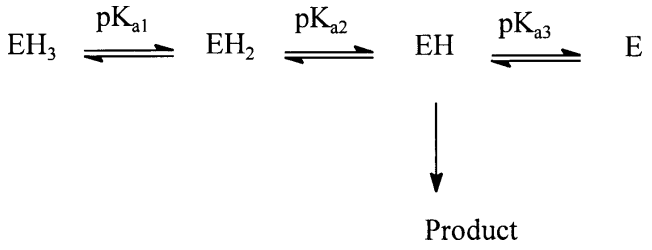


Figure 4 Dependence on pH for the β-lactamase-catalysed hydrolysis of cephaloridine

The pH dependence of log k_{cat}/K_m for the β-lactamase-catalysed hydrolysis of cephaloridine at 30 °C and constant ionic strength of 1.0 M (NaCl). The solid line was calculated using eqn. (2) in the text.



Scheme 2

and not because of acid-catalysed inactivation–degradation of the protein. Repeat hydrolysis reactions, brought about by injecting a second sample of substrate into the reaction mixture after the completion of hydrolysis of the first aliquot, gave identical pseudo-first-order rate constants. Furthermore, leaving the enzyme at pH 4.0 for the period of the kinetic study and then changing the pH to 7.0, resulted in rate constants identical with those obtained directly at neutral pH. There was no evidence for rapid and reversible denaturation of the enzyme at pH 4.5 from changes in the CD spectrum.

It appears therefore that the rate is suppressed at lower pH because of two protonation processes. Similar behaviour was observed for the two substrates, a penicillin and a cephalosporin, and this unusual behaviour was attributed to protonation of the enzyme. The pK_a values corresponding to these two equilibrium processes, i.e. pK_{a1} and pK_{a2} in Scheme 2, must be similar. The data does not justify trying to separate these and the lines in Figure 3 and Figure 4 are generated from eqn. (2), with $pK_{a1} = pK_{a2} = 5.60 \pm 0.20$ and $pK_{a3} = 9.50 \pm 0.20$.

$$k_{obs} = \frac{k^{max}}{(H^+)/K_{a2} + (H^+)^2/K_{a1}K_{a2} + 1 + K_{a3}/(H^+)} \quad (2)$$

Kinetic solvent isotope effects

The pH dependence of k_{cat} and k_{cat}/K_m was also studied in ^2H_2O. At pH below 5 the values of k_{cat}/K_m again showed an inverse second-order dependence on hydrogen-ion concentration. The slope of log k_{cat}/K_m against p^{2}H was 2.0. The transfer from H_2O to ^2H_2O must have similar effects on pK_{a1} and pK_{a2} and the data were again insufficiently accurate to separate them. The kinetic solvent isotope effect on k_{cat}/K_m was 1.82 for benzylpenicillin as substrate and 0.85 for cephaloridine as substrate. Cefuroxime is a substrate with a very low K_m, which enables the determination of k_{cat} to be particularly accurate. The kinetic solvent isotope effect $k_{cat}^{H_2O}/k_{cat}^{^2H_2O}$ was 1.5. The derived kinetic parameters in H_2O and ^2H_2O are shown in Table 2.

Table 2 Kinetic parameters for *B. cereus* β-lactamase class B activity

Kinetic parameters for the *B. cereus* β-lactamase class B catalysed hydrolysis of β-lactams in water at 30 °C, $I = 1.0$ M (NaCl).

	Benzylpenicillin	Cephaloridine	Cefuroxime
$pK_{a1} = pK_{a2}$ (H_2O)	5.83 ± 0.05	5.46 ± 0.05	
$pK_{a1} = pK_{a2}$ (^2H_2O)	5.91 ± 0.05	5.70 ± 0.05	
pK_{a3} (H_2O)	9.30 ± 0.1	9.64 ± 0.1	
pK_{a3} (^2H_2O)	10.04 ± 0.1	10.14 ± 0.1	
(k_{cat}/K_m) H_2O ($M^{-1} \cdot s^{-1}$)	4.27 (± 0.6) × 10^5	6.76 (± 0.4) × 10^4	7.74 × 10^5
(k_{cat}/K_m) ^2H_2O ($M^{-1} \cdot s^{-1}$)	2.34 (± 0.5) × 10^5	7.94 (± 0.05) × 10^4)	6.31 (± 0.7) × 10^5
$\frac{(k_{cat}/K_m)\,H_2O}{(k_{cat}/K_m)\,^2H_2O}$	1.82	0.85	1.23
$k_{cat}^{H_2O}$ (s^{-1})	336 ± 10	638 ± 100	35 ± 5
$k_{cat}^{^2H_2O}$ (s^{-1})	232 ± 10	423 ± 78	22 ± 3
$k_{cat}^{H_2O}/k_{cat}^{H_2O}$	1.4	1.5	1.6

Table 3 Effect of methanol concentration on *B. cereus* β-lactamase class B activity

The effect of methanol concentration on the *B. cereus* β-lactamase class B (5.0×10^{-8} M) catalysed by hydrolysis of cefuroxime (2.9×10^{-4} M) in Mops buffer (0.05 M) at pH 7.0, maintained at constant ionic strength of 1.0 M (NaCl) at 30 °C.

Methanol concentration (M)	k_{cat} (s^{-1})
0.00	29.0
0.50	29.1
0.75	28.3
1.00	28.9

Attempted methanolysis

A possible mechanism of action for the metallo-β-lactamase is the formation of an anhydride intermediate as a result of nucleophilic attack of aspartate on the β-lactam carbonyl (see Scheme 7). Such an anhydride could be trapped with methanol to form an ester. However, there was no significant effect of methanol concentration on the value of k_{cat} for the enzyme-catalysed hydrolysis of cefuroxime (Table 3), nor could any ester product be detected. The effect on k_{cat} was investigated because if the rate of anhydride formation was faster than its breakdown, then k_{cat} would reflect this slower rate and could be affected by the methanol concentration. If the anhydride does not accumulate, then k_{cat} would reflect the rate of formation of the anhydride and would be unaffected by additional methanol. The second-order rate constant, k_{cat}/K_m, is likely to reflect the rate of anhydride formation only, irrespective of the relative rates of its formation and breakdown. For this reason, a substrate with a low K_m was chosen for study.

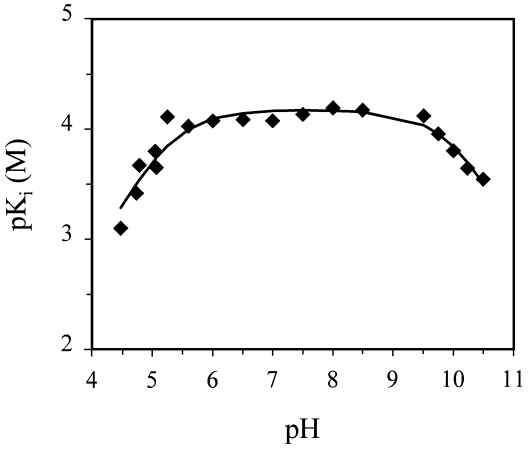
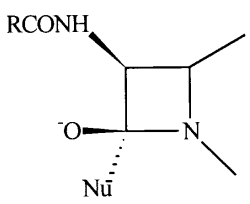


Figure 5 Dependence on pH for β-lactamase of the inhibition of by thiol (Structure 2)

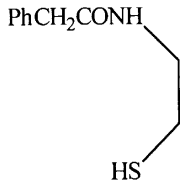
The pH-dependence of pK_i for *B. cereus* β-lactamase class B inhibited by the thiol (Structure 2) at 30 °C and / 1.0 M (NaCl). The values of pK_i were determined using cephaloridine as the substrate.

Inhibition with thiols and pH dependence of K_i

Whatever the detailed mechanism of action of β-lactamase, it is likely that zinc(II) stabilizes the tetrahedral intermediate (Structure 1) presumed to be formed during the catalytic process. We therefore prepared the simple thiol (Structure 2) to see if it could act as an inhibitor, based on the assumption that it possessed sufficient recognition to bind to the active-site zinc(II). The thiol (Structure 2) was indeed a competitive inhibitor of the class B β-lactamase and values of K_i were determined by the effect of varying concentrations of the thiol on the value of the second-order rate constant k_{cat}/K_m for the hydrolysis of benzylpenicillin and cephaloridine.



Structure 1



Structure 2

Inhibition constants, K_i, are true equilibrium constants representing binding of the inhibitor and the enzyme. They have the advantage over kinetic K_m values, which are often complex quantities incorporating rate constants for several of the multiple steps in the enzyme-catalysed reaction [35,36].

Figure 5 shows a plot of the logarithm of the inhibition constant, pK_i, against pH for the class B β-lactamase catalysed hydrolysis of cephaloridine. A very similar plot was observed using benzylpenicillin as substrate (results not shown). Binding was pH independent between pH 6 and 9 but decreased in both acidic and basic solution. The inflections were very similar to those observed for enzyme-catalysed hydrolysis (Figure 3 and Figure 4), with calculated $pK_{a1} = 5.66 \pm 0.4$ and $pK_{a3} = 9.33 \pm 0.1$. The implication is that the ionization states of the enzyme required for catalysis are also those required for binding the inhibitor. However, the slope on the acidic limb was 1.0 in contrast with that observed for hydrolysis, which implies that only one acidic group in the enzyme has an effect on binding the inhibitor.

DISCUSSION

The number of binding sites for zinc(II) per molecule of class B β-lactamases appears to be one [24] or two [25–28]. The crystal structure of the enzyme from *B. cereus* 569/H, used in this study, has one tightly bound zinc [24]. The effect of additional zinc(II) in solution on the catalytic activity of the enzyme at pH 7.0 is minimal (Table 1). A 10^4-fold excess of zinc(II) increased k_{cat}/K_m by less than 36 %, indicating that, for this enzyme at least, there was no catalytically important second site for zinc(II).

Enzyme activity was dependent on both ionic strength and buffer concentration (Figure 1 and Figure 2 respectively) and these parameters obviously need to be controlled especially when only small changes in rates are observed.

The major objectives of the present study were to determine the pK_a of the zinc-bound water in class B β-lactamases and to comment on the mechanism of bond making and breaking in the hydrolysis of β-lactams. There are three particularly relevant observations reported in this study. (i) The inverse second-order dependence of k_{cat}/K_m upon hydrogen ion concentration below pH 5, as shown graphically in the plot of log k_{cat}/K_m against pH (Figure 3 and Figure 4); (ii) the relatively small kinetic solvent isotope effect, $(k_{cat}/K_m)^{H_2O}/(k_{cat}/K_m)^{^2H_2O}$ of 0.85–1.82 and $k_{cat}^{H_2O}/k_{cat}^{^2H_2O}$ of 1.5 (Table 2); (iii) the pH independence of K_i for the inhibition of class B β-lactamase by the thiol (Structure 2) between pH 6 and 9.

pH rate profile

The remarkable feature of the pH-rate profiles given in Figure 3 and Figure 4 is the slope of 2 on the acidic limb, indicating that there are two acidic groups on the low pK_a side which control activity. These two groups have very similar pK_a values, indistinguishable from 5.6. Altogether there are therefore three kinetically important ionizing groups, including one with a pK_a of 9.5 (Scheme 2). It is not known what these ionizations correspond to at the molecular level but identifying the nature of these apparent pK_a values are central to the understanding of the mechanism of action of the metallo-β-lactamases. The apparent pK_a could reflect the ionization of specific groups but it could also result from a change in the rate-limiting step or be a composite constant. The value reported for the pH dependence of the inhibition constant, K_i, for the thiol inhibitor (Structure 2) indicated that the apparent pK_a at 5.6 did correspond to the ionization of a specific functional group.

The pH dependence of k_{cat}/K_m and the associated ionizations, pK_{a1}, pK_{a2} and pK_{a3} (Scheme 2) do not necessarily indicate the mechanistically important form of the catalytic groups but simply give information about the stoichiometry of the transition state. The mechanism may not necessarily involve the two groups of pK_a 5.6 and that of pK_a 9.5 in their deprotonated and acidic forms respectively. The kinetics indicate that there are three basic residues and a proton controlling activity, the position of the proton in the transition state is not known.

Two of the most likely candidates for these ionizations are the zinc-bound water molecule and Asp-90. The zinc of *B. cereus* β-lactamase is co-ordinated to three protein ligands, His-86, His-88 and His-149 and a water molecule [24]. Formally, either the pK_a of 5.6 or 9.5 could conceivably correspond to the ionization of zinc-bound water. The pK_a of hydrated Zn^{2+} is 9.5 [37] but the enzyme environment and the nitrogen ligands could modify this. For example, the pK_a of zinc-bound water in carbonic anhydrase is 6.8 [38,39] and in carboxypeptidase A it is 6.2 [40–43]. The

most likely amino acid candidate for the pK_a of 5.6 is Asp-90 which is highly conserved [24,32].

Identification of the pK_a of Zn(II)-bound water

The pK_a of the zinc(II)-bound water almost certainly corresponds to the high or low pK_a indicated in the pH–k_{cat}/K_m profile (Figure 3 and Figure 4) i.e. 5.6 or 9.5. The pH-independent binding of the thiol inhibitor (Structure 2) between pH 6 and 9 (Figure 5) indicates that it is the low value of 5.6. Scheme 3 shows the possible modes of binding.

$$EZn.OH_2 + RSH \rightleftharpoons EZn.SR + H_3O^+ \quad (3)$$

$$EZn.OH_2 + RS^- \rightleftharpoons EZn.SR + H_2O \quad (4)$$

$$EZn.OH + RSH \rightleftharpoons EZn.SR + H_2O \quad (5)$$

Scheme 3 Possible modes of thiol binding to the metalloenzyme

The pK_a of the thiol (Structure 2) is 9.5, so the predominant species at lower pH is the undissociated neutral form. If the zinc-bound water corresponded to the higher pK_a of 9.5, it would also be undissociated below pH 9 and binding of the neutral thiol would be pH-dependent because of the liberation of the hydronium ion (eqn. 3). Similarly the direct binding of the thiolate anion at a pH below its pK_a would also be pH dependent because the thiolate anion concentration would be proportional to the pH (eqn. 4). If the zinc-bound water had a pK_a of 5.6 it would be fully deprotonated above pH 6. Finally, the only scheme giving pH-independent binding between pH 6 and 9 would be if the neutral undissociated thiol binds to the species of enzyme in which the water bound to the zinc is fully deprotonated (eqn. 5), i.e. the pK_a of the zinc-bound water must be 5.6.

The role of zinc(II)

Information about the mechanism of hydrolysis catalysed by the metallo-β-lactamase may be gained by comparison with the more studied carboxypeptidase A and carbonic anhydrase. Carboxypeptidase A is a zinc metalloprotease but, despite intense mechanistic studies, the detailed roles of the catalytic groups remain controversial [44–47]. The hydratase and esterase activities of carbonic anhydrase mutants suggest that the zinc(II) co-ordinates to the carbonyl oxygen and stabilizes the negative charge developed on this oxygen following nucleophilic attack. Stabilization of the tetrahedral intermediate anion by these enzymes appears to be more important than the nucleophilicity of the zinc hydroxide [48–50].

Is it better for catalysis to have a higher or lower pK_a zinc-bound water? By definition, the better the Lewis acid in stabilizing the negative charge developed on the tetrahedral intermediate from the β-lactam carbonyl oxygen, the greater also will be the effect on the acidity of bound water, i.e. the lower the pK_a. The lower the pK_a of zinc-bound water the more 'tightly bound' is the resulting hydroxide ion, which also becomes the dominant species even at lower pH. It makes evolutionary sense for the pK_a of the zinc-bound water to be just below the normal pH required for catalytic hydrolysis. If the pK_a is too low the hydroxide ion is the dominant species but will be tightly bound to the zinc, and a low pK_a corresponds to a more weakly nucleophilic hydroxide ion. If the pK_a is too high, metal co-ordinated water will be the dominant species but deprotonation will give a more nucleophilic zinc-bound hydroxide and general base-catalysed proton removal would become necessary. Similarly a high pK_a water implies a weak Lewis acid and therefore the zinc ion will be less efficient at stabilizing the tetrahedral intermediate.

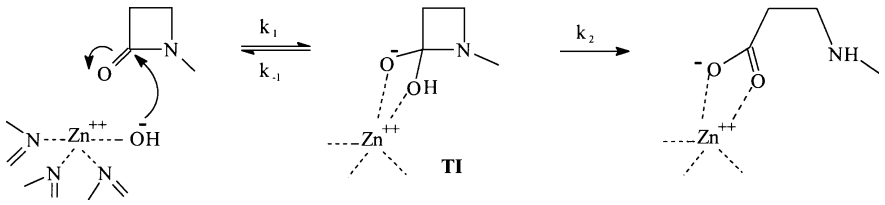
Factors which make zinc(II)-bound water more acidic than bulk water also decrease the basicity of the metal-bound hydroxide ion. Consequently, breaking the newly formed bond between the substrate carbonyl C and the O of the attacking nucleophile in the tetrahedral intermediate (TI) to regenerate the β-lactam substrate involves the expulsion of a reasonably good leaving group. Therefore it would not be surprising if the rate-limiting step is not formation of the tetrahedral intermediate (k_1) but rather its breakdown (k_2), as $k_{-1} \gg k_2$ (Scheme 4).

Rate-limiting breakdown of tetrahedral intermediates formed during nucleophilic substitution of the β-lactam antibiotics is often observed. For example, the Brønsted β_{nuc} values and the kinetic solvent isotope effect observed in the alcoholysis of benzylpenicillin by alkoxide ions suggest that C–N fission coupled with proton transfer is the rate-limiting step [51].

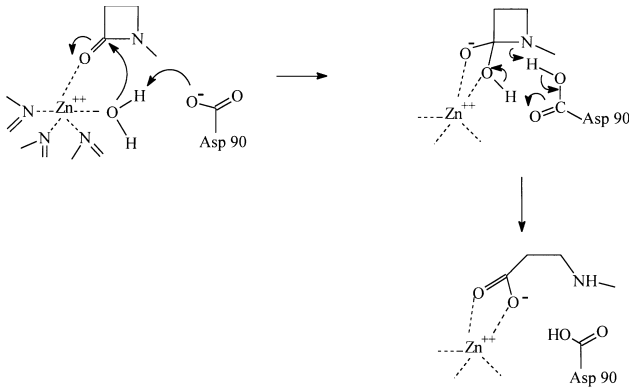
It therefore seems probable that the mechanism for the zinc-β-lactamase-catalysed hydrolysis of β-lactams involves initial attack by zinc(II)-bound hydroxide ion followed by rate-limiting breakdown of the tetrahedral intermediate. A major role of zinc(II) is the stabilization of the oxyanion of the tetrahedral intermediate (TI) (Scheme 4), and, as proposed below, the subsequent stabilization of the dianion.

The role of Asp-90

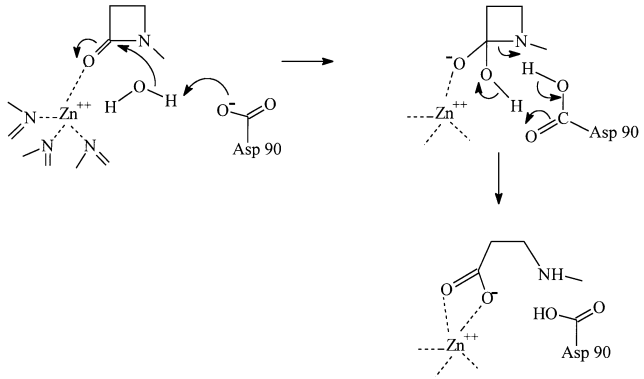
Similar to β-lactamase, there is an essential carboxylate residue in the active site of carboxypeptidase but rather than an aspartate it is a glutamate residue (Glu-270), but its role in either enzyme is not known. With normal peptide substrates the carboxylate group of carboxypeptidase is thought to act as a general base catalyst to remove a proton from either zinc-bound water or unco-ordinated water [52,53]. The equivalent mechanisms for β-lactamase are shown in Scheme 5 and Scheme 6. The pH rate profiles (Figure 3 and Figure 4) may at first appear consistent with the carboxylic acid being in its ionized form for activity and perhaps acting, as previously suggested [30], as a general base catalyst by removing a proton from the zinc(II)-bound water



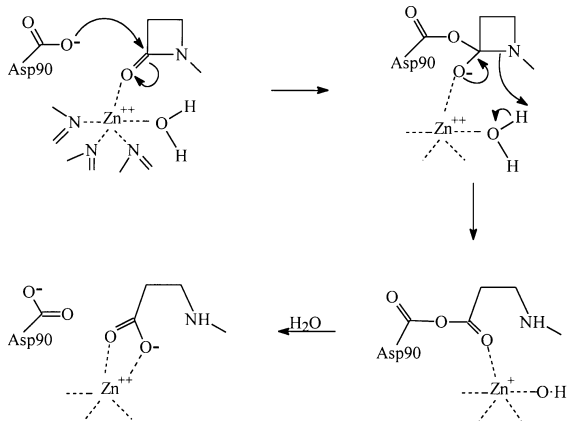
Scheme 4 Possible reversible formation of the tetrahedrial intermediate



Scheme 5 General base-acid catalysis and attack by zinc-bound water



Scheme 6 General base catalysis by Asp-90



Scheme 7 Nucleophilic catalysis by Asp-90

(Scheme 5). However, there are several difficulties with accepting this mechanism.

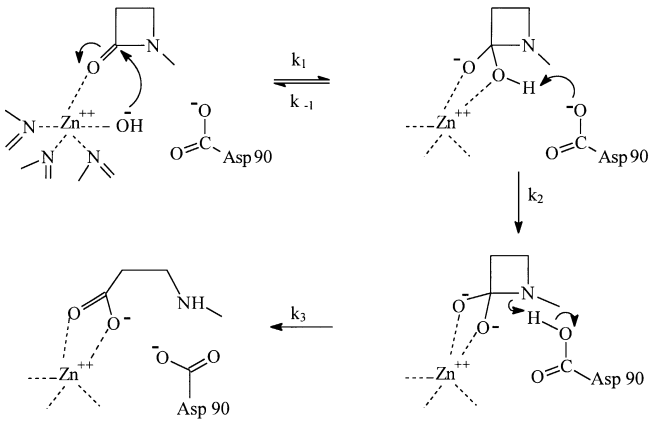
A major problem with the widely accepted mechanism of general base-catalysed removal of a proton from zinc(II)-bound water in a process which is concerted with nucleophilic attack on the β-lactam–amide carbonyl group (Scheme 5) is the relative acidity of this proton. Even if the pK_a of the zinc(II)-bound water is about 9, then 10% of the species already exist in the fully deprotonated form at pH 8 and 1% at pH 7. Presumably, the deprotonated form is a much better nucleophile than the species which is only partially deprotonated. There is little or no catalytic advantage in having a general base remove a proton when the more active species is already present! One could argue that deprotonation is slow and that the enzyme has developed to facilitate this process. However, even the non-enzyme-catalysed deprotonation rate for an acid pK_a 9 is approx. 10^3 s^{-1} by hydroxide ions at pH 7 and is even faster by buffer species. Therefore, irrespective of whether the pK_a of the zinc-bound water is 5.6 or 9.5, it appears more logical to assume that nucleophilic attack on the β-lactam–amide carbonyl to form the tetrahedral intermediate occurs from zinc(II)-bound hydroxide ion. However, as discussed above, this may not be the rate-limiting step. Although C–N bond fission is the most energetically difficult process in amide hydrolysis, little attention is normally given to the mechanism of the breakdown of the tetrahedral intermediate. It is usually assumed that, in carboxypeptidase A, the undissociated carboxylic acid of Glu-270 acts as a general acid catalyst protonating the amine nitrogen leaving group to facilitate C–N bond fission [54]. The timing of these proton transfer steps is not known but, for convenience, in Schemes 6 and 7, deprotonation of the hydroxy group which attacked the carbonyl carbon is shown to occur simultaneously with proton transfer to nitrogen.

Both mechanisms shown in Schemes 5 and 6 involve significant proton transfer steps. Whether formation or breakdown of the tetrahedral intermediate is rate-limiting, a significant kinetic solvent isotope effect would be expected, which is not observed.

Another possible role for Asp-90 is as a nucleophile with zinc(II) stabilizing the tetrahedral intermediate (Scheme 7). Breakdown of the tetrahedral intermediate could be facilitated by zinc-bound water as a general acid catalyst. This mechanism generates a mixed carboxylic acid anhydride as an intermediate, which is subsequently hydrolysed by water (Scheme 7). Nucleophilic catalysis and the anhydride mechanism have often been proposed as the pathway adopted by the carboxypeptidase A-catalysed hydrolysis of active substrates, such as esters [55–59]. It is interesting to note that the reactivity of the β-lactam of penicillins is similar to that of a simple alkyl ester rather than a normal peptide or amide [51,60–63]. Attempts to trap the anhydride with methanol were not successful and, at present, there is little evidence to support this mechanism.

Proposed mechanism

The mechanism which is compatible with the crucial observations reported here, two catalytically important groups of pK_a approx. 5.6 each required in their deprotonated forms and the lack of a significant kinetic solvent isotope effect, is shown in Scheme 8. Zinc(II)-bound water is present at neutral pH in its deprotonated form and acts directly as the nucleophile to attack the β-lactam carbonyl. Zinc(II) also acts as an electrophile to stabilize the negative charge generated on the carbonyl oxygen on forming the tetrahedral intermediate [64]. Ring opening of the β-lactam ring is not a facile process and this first step (k_1 of Scheme 8) is likely to be reversible, i.e. collapse of the tetrahedral intermediate to regenerate the β-lactam occurs at a faster rate than C–N bond fission ($k_{-1} \gg k_2$, as shown in Scheme 8). This is not surprising as carbon–oxygen bond fission expels a reasonable leaving group, the zinc-bound hydroxide ion, the conjugate acid of which has a



Scheme 8 Nucleophilic attack by zinc-bound hydroxide

pK_a of 5.6. Carbon–nitrogen bond fission to expel the amine leaving group will not occur without protonation of nitrogen. In amide hydrolysis, carbon–nitrogen bond fission is sometimes facilitated by deprotonation of the tetrahedral intermediate to generate a dianionic tetrahedral intermediate [65–69]. This dianion generates a system with more 'electron-push'. In the metallo-β-lactamase mechanism this process could occur by the carboxylate anion of Asp-90 (Scheme 8) to give a dianionic system with two negatively charged oxygens bound to zinc. The formation of the dianion of the tetrahedral intermediate nicely explains the requirement for two negative charges in the transition state. Although ionic-strength effects are difficult to interpret directly, those observed here (Figure 1) are consistent with increased charge development in going from the initial to the transition state. Deprotonation of the attacking zinc-bound hydroxide also avoids formation of an unstable system of a neutral undissociated carboxylic acid bound to zinc(II). The final step would then involve general acid-catalysed breakdown of the zinc-bound dianionic tetrahedral intermediate, with the now undissociated Asp-90 donating a proton to the departing amine nitrogen (Scheme 8). Either k_2 or k_3 could be rate-limiting. The first step is expected to show an inverse kinetic solvent isotope effect which would be counterbalanced by the primary effect associated with the k_2 and k_3 steps. Hence, the experimental observation of small solvent kinetic isotope effects of 0.85 for cephaloridine and 1.82 for benzylpenicillin. This mechanism also nicely explains the requirement for both zinc-bound water and Asp-90 to be in their deprotonated forms and, hence, accounts for the decrease in k_{cat}/K_m with the second power in hydrogen-ion concentration below pH 5.

The kinetic solvent isotope effects are compatible with this mechanism [70]. The first step, attack of hydroxide-ion-bound zinc on the β-lactam carbonyl is expected to show an isotope effect $k_{H_2O}/k_{^2H_2O}$ of 0.72 ± 0.05 [70–73]. The second step, if rate limiting, would generate a normal isotope effect of 2.5 ± 0.5 [70] giving a predicted observed value of 1.8 ± 0.5. A similar value would be expected if breakdown of the dianionic tetrahedral intermediate was rate limiting. The observed value of $k_{cat}^{H_2O}/k_{cat}^{^2H_2O}$ of 1.5 ± 0.1 is therefore compatible with the proposed mechanism (Scheme 8).

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