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

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The plot of $k_{\text{cat}}/K_m$ 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 $k_{\text{cat}}/K_m$ is 1.3 ± 0.5 and that on $k_{\text{cat}}$ 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 diatomic 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 diatomic 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](image)

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 1H-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 acid that protonated the oxygen anion of the tetrahedral intermediate which is stabilized by the zinc ion. It was originally proposed that Glu-37 acted as a general acid-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-lactamases 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-lactamases 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 pKₐ of the zinc-bound water.

**EXPERIMENTAL**

**Synthesis of N-(2-mercaptopethyl)-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 Na₂SO₄. The solvent was removed by rotary evaporation to give a white product. The total yield was 87%.

Some characteristics of the thiol were: IR (Nujol), 3253 (amide NH), 2544 (SH) and 1637 cm⁻¹ (amide CO); ¹H-NMR, J values are given in Hz, δH (C6DCl₃) 7.30 (5 H, m, Ar-H), 5.75 (1 H, NH), 3.55 (2 H, s, CH₂-C₆H₄), 3.35 (2 H, q, J 6.6, H-1'), 2.52 (2 H, dt, J 6.6 and 8.5, H-2').

A pH 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), hexafluorosopropanol (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 0.04–20 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 kcat/Km. Inhibition constants were calculated using the equation (eqn. 1) for competitive inhibition:

$$\text{Rate} = \frac{k_{cat} [E] [S]}{[S] + K_{cat} \left(\frac{K_I + [I]}{K_I}\right)}$$

(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, pKₐs 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, kobs, were obtained, which were shown to be first-order in enzyme concentration and therefore, the second-order rate constant kcat/Km, was derived by dividing kobs by the concentration of enzyme used.

The effect of ionic strength on the second-order rate constant kcat/Km 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 kcat/Km about two-fold. The ionic strength effect...
buffers decrease the rate of hydrolysis; 0 changes in the buffer concentration. Both Mops and phosphate compensate for changes in ionic strength brought about by strength of 1 cephaloridine in a solution maintained at a constant total ionic and buffer type on the rate of the enzyme-catalysed hydrolysis of cephaloridine 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 \(k_{\text{cat}}/K_m\) 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.

was slightly greater when phosphate buffer was compared with Mops.

Figure 2 Dependence on buffer concentration for the \(\beta\)-lactamase-catalysed hydrolysis of cephaloridine
The dependence of the second-order rate constant \(k_{\text{cat}}/K_m\) for the \(\beta\)-lactamase II catalysed hydrolysis of cephaloridine upon buffer concentration (○, Mops; +, phosphate) at a constant buffer concentration. The ionic strength was kept constant throughout at 1.0 M by varying the concentration of NaCl.

Table 1 Effect of zinc(II) concentration on \(B.\) cereus \(\beta\)-lactamase class B activity
The effect of zinc(II) concentration on the \(B.\) cereus \(\beta\)-lactamase class B (1.0 \times 10^{-7} M) catalysed hydrolysis of cephaloridine (1.52 \times 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.

<table>
<thead>
<tr>
<th>Zn(II) concentration (M)</th>
<th>(10^{-4} \times k_{\text{cat}}/K_m) (M^{-1} s^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 \times 10^{-7}</td>
<td>7.54</td>
</tr>
<tr>
<td>2.0 \times 10^{-7}</td>
<td>7.96</td>
</tr>
<tr>
<td>1.1 \times 10^{-4}</td>
<td>7.39</td>
</tr>
<tr>
<td>1.0 \times 10^{-4}</td>
<td>8.42</td>
</tr>
<tr>
<td>1.0 \times 10^{-4}</td>
<td>8.19</td>
</tr>
<tr>
<td>1.0 \times 10^{-3}</td>
<td>10.28</td>
</tr>
</tbody>
</table>

Figure 3 Dependence on pH for the \(\beta\)-lactamase-catalysed hydrolysis of benzylpenicillin
The pH dependence of \(\log k_{\text{cat}}/K_m\) for \(\beta\)-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 \(k_{\text{cat}}/K_m\) on Zn(II) concentration
Some of the metallo-\(\beta\)-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 by varying the concentration of zinc(II). Table 1 shows the values of \(k_{\text{cat}}/K_m\) with zinc(II) concentrations varying from 0.0 to 1.0 \times 10^{-5} M zinc sulphate. The second-order rate constant increased, but by only 36% over a 10-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 \(\beta\)-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, \(k_{\text{cat}}/K_m\), for the hydrolysis of benzylpenicillin and cephaloridine, respectively, catalysed by class B \(\beta\)-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...
The data does not justify trying to separate these and the lines in rapid and reversible denaturation of the enzyme at pH 4 those obtained directly at neutral pH. There was no evidence for the pH dependence of kinetic solvent isotope effects of the enzyme. The pH values corresponding to these two equilibria 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 $pK_{a3} = 9.50 \pm 0.20$.

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

**Kinetic solvent isotope effects**

The pH dependence of $k_{cat}$ and $k_{cat}/K_m$ was also studied in $^2$H$_2$O. 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 pH was 2.0. The transfer from H$_2$O to $^2$H$_2$O 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 $k_{cat}/K_m^{{^2}H}_2O/k_{cat}/K_m$ was 1.5. The derived kinetic parameters in H$_2$O and $^2$H$_2$O are shown in Table 2.

**Table 2  Kinetic parameters for B. cereus $\beta$-lactam class B activity**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Benzylopenicillin</th>
<th>Cephaloridine</th>
<th>Cefuroxime</th>
</tr>
</thead>
<tbody>
<tr>
<td>$pK_{a1}$</td>
<td>$pK_{a2}$</td>
<td>$pK_{a3}$</td>
<td>$pK_{a1}$</td>
</tr>
<tr>
<td>5.60 ± 0.20</td>
<td>9.50 ± 0.20</td>
<td>10.638 ± 0.20</td>
<td>5.51 ± 0.05</td>
</tr>
<tr>
<td>10.638 ± 0.20</td>
<td>10.14 ± 0.1</td>
<td>10.638 ± 0.20</td>
<td>6.76 ± 0.4</td>
</tr>
<tr>
<td>1.82</td>
<td>0.85</td>
<td>1.23</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3  Effect of methanol concentration on B. cereus $\beta$-lactam class B activity**

<table>
<thead>
<tr>
<th>Methanol concentration (M)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>29.0</td>
</tr>
<tr>
<td>0.50</td>
<td>29.1</td>
</tr>
<tr>
<td>0.75</td>
<td>28.3</td>
</tr>
<tr>
<td>1.00</td>
<td>28.9</td>
</tr>
</tbody>
</table>

**Attempted methanolysis**

A possible mechanism of action for the metallo-$\beta$-lactamase is the formation of an anhydride intermediate as a result of nucleophilic attack of aspartate on the $\beta$-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.
Inhibition with thiols and pH dependence of $K_i$

Whatever the detailed mechanism of action of $\beta$-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 $\beta$-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](image1.png)

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 $\beta$-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 $pK_{i1} = 5.66 \pm 0.4$ and $pK_{i2} = 9.33 \pm 0.1$. 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 $\beta$-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 $10^5$-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 $pK_i$ of the zinc-bound water in class B $\beta$-lactamases and to comment on the mechanism of bond making and breaking in the hydrolysis of $\beta$-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, $(k_{cat}/K_m)^H/(k_{cat}/K_m)^D$ of 0.85–1.82 and $k_{cat}/K_m^{H+/D+}$ of 1.5 (Table 2); (iii) the pH independence of $K_i$ for the inhibition of class B $\beta$-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-$\beta$-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, $pK_a$, $pK_{i1}$ and $pK_{i2}$ (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 $\beta$-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 $5.6$. The $pH$-independent binding of the thiolate anion at a $pH$ below its $pK_a$ 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 $pH$-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 carboxylic anhydride. 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 carboxyl 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 $k_1 >> k_2$ (Scheme 4).

Rate-limiting breakdown of tetrahedral intermediates formed during nucleophilic substitution of the β-lactam antibiotics is often observed. For example, the Bronsted $pK_a$ values and the kinetic solvent isotope effect observed in the alcoholysis of benzylpenicillin by alkoxy 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 uncoordinated 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 tetrahedral intermediate](image)
Catalytic mechanism of β-lactamase

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 pKa 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 pKa 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 pKa 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 pKa 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 (k1, 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 ≫ k2, 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
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 di-anion generates a system with more ‘electron-push’. In the metallo-β-lactamase mechanism, this process could occur by the carboxylic anion of Asp-90 (Scheme 8) to give a dianionic zinc-bound tetrahedral intermediate, with the now undissociated carboxylate anion of Asp-90 (Scheme 8) to give a dianionic zinc-bound hydroxide

\[ \text{Asp-90}^{-2} \]

or

\[ \text{Asp-90}^{-2} \]

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