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FEATURE ARTICLE

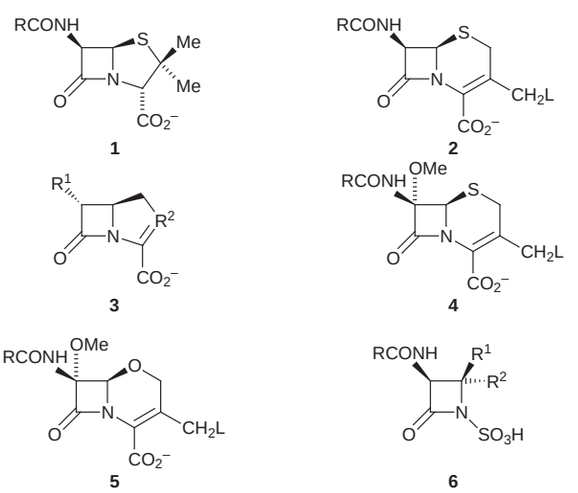
The mechanism of catalysis and the inhibition of β-lactamases

Michael I. Page*† and Andrew P. Laws

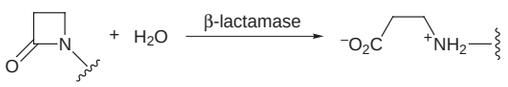
Department of Chemical and Biological Sciences, The University of Huddersfield, Queensgate, Huddersfield, UK HD1 3DH

Formation of a tetrahedral intermediate by nucleophilic attack on the β-lactam carbonyl carbon of penicillins generates a lone pair on the β-lactam nitrogen which is *syn* to the incoming nucleophile, in contrast to the normal *anti* arrangement found in peptides. Ring opening of the β-lactam requires protonation of the β-lactam nitrogen by a general acid catalyst. The general acid/base catalyst in β-lactamases is probably a glutamate and a tyrosine residue in class A and C enzymes, respectively. Phosphonamidates inactivate class C β-lactamases by phosphonylation of the active site serine, the rate of which is enhanced by a factor of at least 10^6. The enzyme's catalytic machinery used for hydrolysis is also used for phosphonylation. The rate enhancement may be greater than 10^9 if the mechanism occurs by an inhibitor assisted reaction involving intramolecular general acid catalysis. Class B metallo-β-lactamases are inhibited by thiol derivatives with K_i as low as 10 μM. The mechanism of hydrolysis of the metallo-β-lactamase involves a dianionic tetrahedral intermediate stabilised by zinc(II).

β-Lactam antibiotics account for 50% of the world's total antibiotic market. They have high anti-bacterial activity and low toxicity.[1] In addition to the traditional families of penicillins and



cephalosporins—the penams **1** and cephems **2**, respectively—there are the carbapenems **3**, the cephamycins **4**, the oxacephamycins **5** and the monobactams **6**.[2] This battalion of structures differ in their spectrum of antibacterial activity—from narrow anti-*Staphylococcal* agents to broad spectrum β-lactams capable of killing a wide variety of Gram positive and negative bacteria—and in their ability not to be susceptible to the β-lactamase hydrolytic enzymes which are the most common, and growing, form of bacterial resistance to their normally lethal action. β-Lactamases catalyse the hydrolysis of the β-lactam to give the ring opened and bacterially inert β-amino acid (Scheme 1).[3]



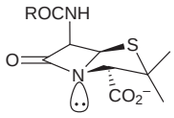
Scheme 1

Two main therapeutic strategies have been adopted to counteract bacterial resistance to β-lactam antibiotics. One involves the design of antibiotics which are not susceptible to β-lactamase catalysed hydrolysis. The other is to use an inhibitor or inactivator of β-lactamase together with a normal β-lactam antibiotic. Unfortunately, bacteria seem to be able to produce new β-lactamases which catalyse the hydrolysis of previously poor substrates and which are no longer susceptible to previous inhibitors. For example, when the carbapenems **3**, such as imipenem, were first introduced in the 1970s they were seen as versatile broad-spectrum antibacterials resistant to hydrolysis by most β-lactamases. However, now 'carbapenamases' are increasingly produced by a variety of bacteria.[4] There are two distinct types of β-lactamase production in bacteria and so far about 200 different enzymes have been identified.[5] Inducible enzymes may be chromosomal or plasmid encoded but the more common constitutive β-lactamase production is predominantly plasmid mediated. Constitutive mutants of Gram-negative strains produce enormous amounts of enzyme so that periplasmic β-lactamase concentrations[6] may be up to 1 mM—about 10^4-fold greater than that used in most laboratory kinetic experiments. Such high concentrations of catalyst combined with their high efficiency means that there is little chance of β-lactam antibiotics reaching their targets without being hydrolysed.

Chemical reactivity of β-lactams

Four membered β-lactams occur relatively rarely in nature, therefore it is not surprising that the biological activity of these compounds should be attributed to the chemical reactivity of the β-lactam ring. Shortly after the introduction of penicillin to the medical world it was suggested that the antibiotic's activity was due to the inherent strain of the four-membered ring or to reduced amide resonance.[7] The non-planar butterfly shape of the penicillin molecule **7** could reduce amide resonance and thus increase the susceptibility of the carbonyl group to nucleophilic attack, compared with planar amides. However, there is little evidence to suggest that the kinetic reactivity of β-lactams in penicillins and cephalosporins is due to an unusually strained or an amide-resonance inhibited β-lactam.[2] Interestingly, although the rate of alkaline hydrolysis of the simple β-lactam **8** is only three-fold greater than that of **9**,[2] the corresponding 4-membered β-sultams[8] and β-phospholactams[9] are 10^7–10^9 fold more reactive than the corresponding acyclic sulfonamides and phosphonamidates, respectively.

Nucleophilic substitution at the carbonyl of β-lactams is an acyl transfer process involving covalent bond formation between the carbonyl carbon and the nucleophile and C–N bond fission of the β-lactam (Scheme 2). In these types of reactions the mechanism involves, at least, a two-step process.[2,7] Covalent bond formation to the incoming nucleophile occurs before C–N bond fission, resulting in the reversible formation of



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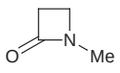

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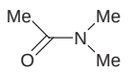

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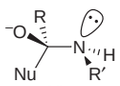

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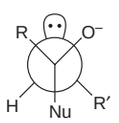

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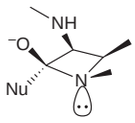

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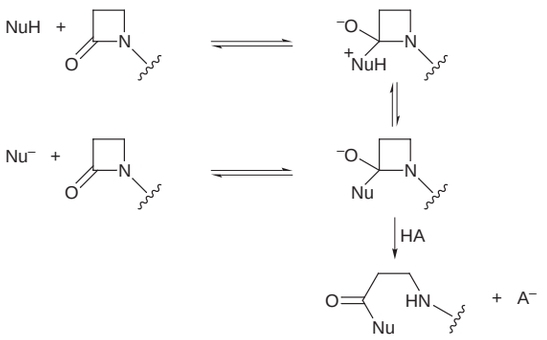
a tetrahedral intermediate. Contrary to expectations, opening the four-membered ring is not a facile process.[10] In many of these nucleophilic substitution reactions the rate-limiting step is often not the first addition step but a subsequent one which may sometimes even be ring-opening itself.[2]

Those reactions which involve the attack of a neutral nucleophile which has an ionisable hydrogen (Scheme 2) invariably require general base catalysis to remove the proton.[2,7] The requirement for proton removal is paramount—and in extreme cases only the reaction of the anionic nucleophile is observed (Scheme 2). For example, there is no pH-independent



Scheme 2

reaction of water with penicillin, and alcohols react only through their anions.[11] The importance of general base catalysis is a reflection of the fact that contrary to expectations penicillins are not powerful acylating agents.[11] Similarly, C–N bond fission requires protonation of the amine nitrogen.[2,7]

It has been suggested that ring opening does not occur by stretching of the C–N bond but rather by a rotational motion.[10,12] This minimises strain effects and maximises favourable orbital interactions. The unusual mode of C–N bond fission could have interesting consequences in the enzyme-catalysed hydrolysis of β-lactams for the geometrical relationship of the proton donor in the protein and the amine leaving group.

Another interesting difference between nucleophilic substitution in penicillins and peptides/amides is the preferred direction of attack and the geometry of the initially formed tetrahedral intermediate. It is usually assumed that nucleophilic attack on the carbonyl carbon of a planar peptide will generate a tetrahedral intermediate with the lone pair on nitrogen *anti* to the incoming nucleophile, (**10**, **11**). Conversely, nucleophilic attack on β-lactams occurs from the least hindered α-face (*exo*) so that the β-lactam nitrogen lone pair is *syn* to the incoming nucleophile in the tetrahedral intermediate (**12**).[13, 14] This has obvious consequences for the placement of catalytic groups—particularly the general acid donating a proton to the departing amine of the β-lactam.[12]

β-Lactamases

The main mechanistic division of β-lactamases is into serine enzymes and zinc enzymes.[3] The former have an active site serine residue and the catalytic mechanism involves the formation of an acyl–enzyme intermediate. The metallo-enzymes appear to involve only non-covalently bound intermediates. On the basis of their amino acid sequences, the serine β-lactamases are sub-divided into three classes—A, C and D—whereas the class B β-lactamases consist of the zinc enzymes.[3]

Serine β-lactamases

Structure

The class A and class C enzymes are monomeric medium-sized proteins with M_r values of about 29 000 and 39 000, respectively.[3] The structures of four class A[15–17] and two class C[18,19] β-lactamases have been reported. There are two major structural domains—all-α and α/β—with the active site situated in a groove between the two domains. The class C β-lactamases have additional loops and secondary structure on the all-α domain. The active site serine is situated at the N-terminus of the long, relatively hydrophobic, first α-helix of the all α-domain. There are several highly-conserved residues surrounding the active site which may be involved in substrate recognition and the catalytic process of bond making and breaking. Firstly, a lysine residue is always present as the third amino acid and one helix-turn further down the chain from the serine and pointing into the active site. Two other residues forming one side of the catalytic cavity are Ser-Xaa-Asn (class A) or Tyr-Xaa-Asn (classes C and D) with the side chains of the first and third residues pointing into the active site. The Ser130-Asp131-Asn132 (the SDN loop) motif is also almost invariant in class A. The other side of the cavity is formed from the β-sheet of the α/β domain and is generally Lys-Thr-Gly. In class A enzymes there is also a conserved glutamate which, in the static crystal structure, is hydrogen-bonded to a conserved water molecule which in turn is hydrogen-bonded to the active site serine.

Mechanism of hydrolysis

There is very strong evidence for the formation of an acyl enzyme intermediate (Scheme 3)—including electrospray mass

Scheme 3

spectrometry,[20] infra-red measurements,[21] trapping experiments,[22] the determination of the rate constants for their formation and breakdown[23] and even an X-ray crystal structure.[24]

The two experimental kinetic parameters associated with simple systems exhibiting Michaelis–Menten behaviour are the second-order rate constant k_{cat}/K_m determined below saturation and the first order rate constant k_{cat} determined above saturation.[25] The *interpretation* of these experimental macroscopic rate constants as they vary with parameters such as pH, substituents in the substrate[26,27] and amino acid replacement in the enzyme[28] depends upon the model assumed for the reaction pathway. The overall reaction pathway for the serine β-lactamases is given in eqn. (1).

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EA \xrightarrow{k_3} E + P \qquad (1)$$

It is usually assumed that formation of the usually relatively unstable acyl enzyme (EA) is irreversible, *i.e.* $k_{-2} = 0$ and the second-order rate constant k_{cat}/K_m, determined below saturation

conditions, is then given by eqn. (2) and hence always reflects the rate of acylation *irrespective* of the value of k_2 and k_3.

$$\frac{\text{Rate}}{(\mathbf{E})(\mathbf{S})} = \frac{k_{cat}}{K_m} = \frac{k_1 k_2}{k_{-1} + k_2} \quad (2)$$

Under saturation conditions the observed rate constant, k_{cat}, is independent of substrate concentration and is given by eqn. (3) and so simplifies to the rate of acylation k_2, if $k_3 >> k_2$ and the enzyme is essentially present as the enzyme–substrate complex. Conversely, k_{cat} reflects the rate of deacylation, k_3, when $k_2 >> k_3$ and the enzyme is present mainly as the acyl enzyme.

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3} \quad (3)$$

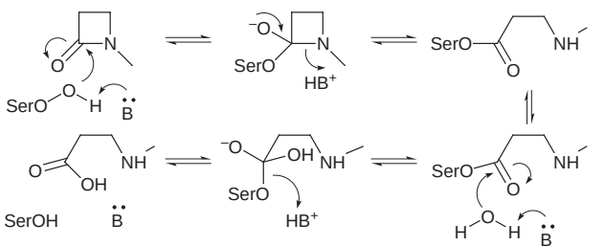
However, if formation of the acyl enzyme is *reversible* then k_{cat}/K_m and k_{cat} are given by eqns. (4) and (5), respectively. For

$$\frac{k_{cat}}{K_m} = \frac{k_1 k_2 k_3}{k_3(k_{-1} + k_2) + k_{-1}k_{-2}} \quad (4)$$

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_{-2} + k_3} \quad (5)$$

serine proteases, k_{-2} represents the 'intermolecular' aminolysis of the acylenzyme to regenerate the peptide substrate. For the hydrolysis of β-lactams catalysed by β-lactamases the k_{-2} step corresponds to 'intramolecular' aminolysis to regenerate the β-lactam substrate (Scheme 3). Although the formation of a strained four-membered β-lactam is likely to have a significant activation energy it is not totally inconceivable that this intramolecular step has an entropic advantage[29] and is competitive with intermolecular hydrolysis of the acylenzyme. For example, we have demonstrated, using esterase enzymes, that lactams can be formed from amino esters in water in preference to the hydrolysis product.[30] The interpretation of the macroscopic rate constant k_{cat}/K_m may then become ambiguous because it reflects all microscopic rate constants up to and including the first effectively irreversible step.

Formation of the acyl enzyme intermediate requires at least two proton transfers—proton removal from the attacking serine and proton donation to the departing β-lactam amine (Scheme 4). Despite the availability of a number of X-ray crystal



Scheme 4

structures of several class A and class C β-lactamases[15–19] and many site-directed mutagenesis studies,[28] the indentity of the catalytic groups involved in these proton transfer steps remain elusive.

In principle, the general base which accepts a proton from the nucleophilic serine is not necessarily the same residue which then acts as a general acid to donate a proton to the departing β-lactam nitrogen. Conceptually this is neater and our prejudices are reinforced by the juxtaposition of the nitrogen lone pair, the serine and the general base/acid being on the same (*exo*) α-face of the substrate in the tetrahedral intermediate **12**. Similarly, the hydrolysis of the acyl enzyme requires two proton transfer steps—from water and to the departing serine—and only our preference for simplicity requires these to be one and the same residue *and* the same as that involved in the acylation step (Scheme 4). This hydrolytic water probably also approaches from the α-face and can be displaced by a 6α- or 7α-substituent in the penicillin or cephalosporin nucleus, respectively, which explains the poor activity of class A β-lactamases against imipenem[31] and cephamycins.[32] Protein stability measurements of covalently-bound phosphonates to a class A β-lactamase also support this hypothesis.[33]

There are therefore at least four transition states corresponding to formation and breakdown of the tetrahedral intermediate to give initially the acyl enzyme followed by its hydrolysis (Scheme 4). The concept of a unique 'transition state analogue' as an inhibitor for an enzyme catalysing a reaction through many steps becomes ambiguous—there could be many analogues corresponding to different transition states.[34] The evolutionarily-perfect enzyme reduces the transition state energies for all steps in the conversion of reactants to products without 'over-stabilising' any intermediates.[35] The maximum second-order rate constant for an enzyme-catalysed reaction is that corresponding to diffusion control (*ca.* 10^8 M^{-1} s^{-1}) and some β-lactamases appear to be near this limit and are 'perfect catalysts'.[36]

In class A β-lactamases there are two serious contenders for the general base/acid—Glu166 and Lys73. The pH-dependence of k_{cat}/K_m indicates two ionising residues are important for catalysis—one of pK_a *ca.* 5 and formally required in its basic form and one of pK_a *ca.* 9 and formally required in its acidic form. If the low pK_a group corresponds to the general base then this is acceptable for the carboxylic acid of Glu166 but is rather low for an ammonium ion of Lys73. Although it has been suggested that Lys73[24,37] may have a reduced pK_a it is difficult to envisage the required reduction of 5.6 pK_a units given the close proximity to Glu166 (2.8 to 3.4 Å).[15–17] A normal pK_a of Lys73 is supported by ^{13}C NMR studies[38] and theoretical calculations.[39] In class A β-lactamases[15–17] the Glu166 carboxylate oxygen distance to Ser70 oxygen is 3.5–4.0 Å whereas the Lys73 nitrogen distance to the Ser70 oxygen is 2.5–2.9 Å. It is possible that the intervening water molecule, seen in the crystal structure, can act as a bridge for proton transfer from Ser70 to Glu166, acting as a general base catalyst. Alternatively, the flexibility of the Ω-loop on which Glu166 resides may allow the distance between the two residues to shorten during the acylation process. The internal structures of proteins are in a state of constant motion at ambient temperature and a degree of flexibility in β-lactamases has been proposed for many years.[40] The ability of β-lactamases to accommodate substrates of diverse structure is indicative of their flexibility.

Site-directed mutagenesis of Glu166 shows that the rates of both acylation and deacylation are affected, although the latter is more so.[28] However, in general base-catalysed reactions the dependence of reactivity upon the basicity of the catalyst and the geometrical relationship between the acid-base pair is not usually strong.[25] It is quite conceivable for mutants to act as reasonable catalysts even when the amino acid residue acting as the general base/acid in the wild type is replaced because another residue, or even the peptide link itself,[14] can take over the role of proton acceptor/donor without significant loss of activity.

On balance it appears that the evidence supports Glu166 (Scheme 4, B = $GluCO_2^-$) acting as the unique proton transfer agent—as a general base and acid in the formation and breakdown of the tetrahedral intermediate, respectively, and in the same role for the hydrolysis of the acyl enzyme intermediate.

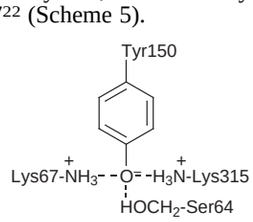
Class C β-lactamases

Historically, class C β-lactamases were often referred to as 'cephalosporinases' because of the characteristic higher turnover numbers, k_{cat}, observed for cephalosporins compared with penicillins. However, the k_{cat}/K_m values for the two classes of enzymes are generally similar and high for both penicillins and

cephalosporins (10^5 to 10^8 M^{-1} s^{-1}).[41] A major difference between the two classes is that for class C β-lactamases deacylation is often rate-limiting, so that the acyl enzyme intermediate may accumulate, giving rise to low values of K_m.[41] Furthermore, class C enzymes are weak catalysts for the hydrolysis of non β-lactam substrates such as esters and thioesters.[42] In class C β-lactamase there is no equivalent glutamate residue but Tyr150 may take its role with Lys67 equivalent to Lys73 in the class A enzyme.[18,19] In addition, it has been suggested that the hydrolytic water involved in deacylation of the acylenzyme approaches from the β-face[43] and that this hydrolysis may be substrate-assisted by the expelled amine, which was the β-lactam nitrogen, acting as a general base catalyst.[44] In class C β-lactamase it has been suggested that the phenol of Tyr150 has a severely reduced pK_a and acts as a general base catalyst for proton removal from Ser64[18,42] although this is not supported by site-directed mutagenesis of Tyr150.[45]

The *E.cloacae* P99 class C β-lactamase catalysed hydrolysis of benzylpenicillin has been shown to yield, in addition to the normal hydrolysis product, the penicilloyl α-methyl ester in the presence of methanol.[22] This is presumably due to the partitioning of the acyl enzyme intermediate between its reaction with water to give the hydrolysis product and its reaction with methanol to produce the penicilloyl ester. At pH 8, with 1 M methanol, up to 30% of the product is the methyl ester. Under saturation conditions the pH-dependence for the second-order rate constant for methanolysis gives two apparent pK_as for the methanolysis reaction which are similar to those observed for hydrolysis, implying that the same catalytically important groups are being used for both reactions.[22]

The absolute values of the pK_a and their shift in D_2O indicate differences between the acidic and basic limbs of the pH–rate profiles. The shift in pK_a^2 in D_2O obtained from k_{cat}/K_m and k_{cat} for hydrolysis is about 0.4 and is that expected from fractionation factors for common acidic groups.[46] However, ΔpK_a^1 is 0.85 from k_{cat}/K_m and k_{cat} for both methanolysis and hydrolysis. This shift and the observed inverse kinetic solvent isotope effect on k_{cat}/K_m is unusual and is indicative of a system with an abnormally low fractionation factor for the basic species formed and/or a high fractionation factor for the protonic state undergoing dissociation.[47] The most likely basic group responsible for the acid limb of the pH–rate profile for both acylation and deacylation is Tyr150, which is hydrogen-bonded to Lys315 and Lys67[22] (Scheme 5).

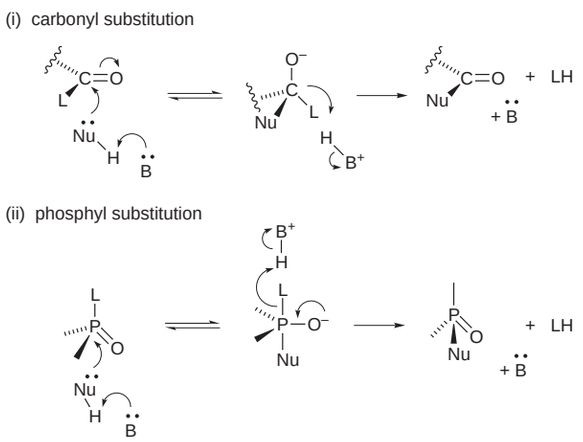


Scheme 5

Because of its relatively positive environment and the strong hydrogen bonding of the phenoxide ion by lysine residues, Tyr150 has a severely reduced pK_a. It appears that the Tyr150 residue is a very strong candidate for the role of a general base catalyst in class C β-lactamases (Scheme 4, B = TyrO$^-$).[22]

Catalytic efficiency of β-lactamase-catalysed acyl and phosphyl transfer

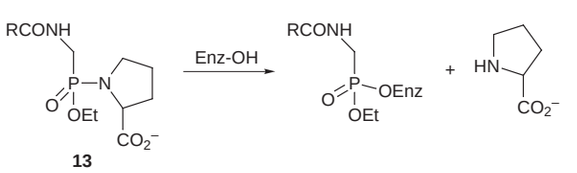
Nucleophilic substitution at acyl centres generally proceeds *via* the formation of an unstable tetrahedral intermediate (TI).[25] The reaction pathway involves a change in geometry as the carbonyl carbon is converted from three- to four-coordination. Furthermore, it is usually assumed that there is some preferential direction of nucleophilic attack such that the incoming nucleophile approaches at approximately the tetrahedral angle to the carbonyl group.[48] By contrast, the associative mechanism for phosphyl group transfer involves a pentacoordinate intermediate with trigonal bipyramidal geometry.[25,49] An initially four-coordinate and tetrahedral phosphorus centre is converted to a five-coordinate one and, in general, it is assumed that the preferential pathway involves the nucleophile taking up the apical position and the leaving group departing from an apical position of the trigonal bipyramidal intermediate (TBPI).[49] Nucleophilic attack at both carbonyl and phosphyl centres is often facilitated by general base catalysis when the nucleophile generates an acidic centre upon covalent bond formation in the intermediate. Similarly if the leaving group is very basic, bond fission and expulsion of the leaving group may be assisted by general acid catalysis. Given the preferential geometrical requirements for incoming nucleophiles and departing leaving groups, there must be a favoured relative positioning of the general base and general acid catalysts (Scheme 6).



Scheme 6

It is often assumed, but with little actual supporting evidence, that enzymes catalyse reactions by an exquisite positioning of the catalytic groups.[50] This is obviously not the case for β-lactamases, which efficiently catalyse the hydrolysis of both penicillins and cephalosporins where there are significant geometrical differences, *e.g.* between the β-lactam carbonyl and nitrogen and the C-3 carboxylic acid in penicillins and that at C4 in cephalosporins. Also if rigid positioning of catalytic groups was so important then it is doubtful if an enzyme with a primary function as a catalyst for acyl transfer could be an effective catalyst for phosphyl transfer because of the geometrical differences in the displacement mechanisms (Scheme 6), although the possibility of pseudorotation in the intermediate may make matters even more complicated.

The class C β-lactamase is, in fact, extremely efficient in enhancing the rate of phosphyl transfer[51] and the pH dependence of the kinetic parameters indicate that similar catalytic machinery is used for both acyl and phosphyl transfer. The class C β-lactamase from *Enterobacter cloacae* P99 is inactivated by the phosphonamidate **13** (R = BnO)[51] by a phosphonylation process analogous to the enzyme-catalysed acylation by the penicillins (Scheme 7).



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Scheme 7

It is necessary to demonstrate that the enzyme actually *enhances* the rate of the phosphonylation reaction. It is well-known that enzymes can catalyse the same reaction of a variety

of substrates and even different reactions with alternative substrates.[52] However, demonstrating the efficiency of the catalysis when the substrate is modified is not straightforward. Modification of the substrate structure can affect the absolute free energies of both the initial reactant state and the transition state, whereas the observed differences in rate constants for the enzyme catalysed reaction only reflect the *difference* in energies between these two states.[53] Different chemical structures can affect the ease of bond-making and -breaking *via* classical electronic factors such as inductive, resonance and steric effects. However, the free energy of activation of an enzyme-catalysed reaction is also affected by the favourable binding energies between the protein and substrate substituents not directly involved with the reaction site. It is therefore necessary to separate these two effects before conclusions about the efficiency of enzyme catalysis can be made. We have suggested[26] that an 'enzyme rate-enhancement factor' (EREF) can be evaluated by dividing the second-order rate constant for the enzyme catalysed reaction, k_{cat}/K_m, by that for hydrolysis of the same substrate catalysed by hydroxide ion, k_{OH}. These factors 'normalise' substrate reactivity and indicate the true efficiency of catalysis.

Both diastereoisomers of the phosphonamidate **13** (R = BnO) completely and irreversibly inactivate the P99 class C β-lactamase in a time-dependent manner to give apparent first order rate constants, k_{obs}, for inactivation. These in turn show a first-order dependence on the concentration of the phosphonamidate to give the second-order rate constants, k_i, for inactivation. The two diastereoisomers show different rate constants for inactivation (5.10 and 0.14 $dm^3\ mol^{-1}\ s^{-1}$). There is no discernible difference in the chemical reactivity of the two diastereoisomers towards alkaline hydrolysis. The 36-fold difference in reactivity is therefore good evidence of selectivity in the reaction of the enzyme with the two phosphonamidates and indicative of specific interactions between the inactivators and the protein. Further evidence of selectivity is seen from the fact that the phosphonamidate **13** (R = BnO) is not a significant inhibitor of either the class A β-lactamase or the class B zinc-dependent β-lactamase from *B. cereus*. The time-dependent inactivation is indicative of covalent bond formation between the enzyme and the phosphonamidate, which is confirmed by electrospray mass spectrometry (ESMS).[51] For the most reactive diastereoisomer, inactivation of the enzyme occurs by formation of a 1 : 1 covalently bound enzyme–inactivator complex in which a proline residue has been displaced by a nucleophilic group on the enzyme—presumably the active site serine. Conversely, the less reactive diastereoisomer reacts with β-lactamase by displacing ethanol, which can be detected by gas chromatography. The observation that the enzyme reacts with one diastereoisomer by displacing proline and with another by displacing ethanol is again indicative of a stereoselective reaction occurring at the active site.

Phosphonamidates are relatively chemically stable and the fast reaction with the β-lactamase indicates that the catalytic machinery of the enzyme used for hydrolysis is also used for phosphonylation and inactivation. This is confirmed by the enzyme rate enhancement factors. The P99 β-lactamase catalyses the hydrolysis of benzylpenicillin with an EREF of 2.6×10^8. Phosphonylation of β-lactamase by the most reactive diastereoisomer of the phosphonamidate **13** occurs by P–N fission and displacement of proline, but the hydroxide ion-catalysed hydrolysis of the phosphonamidate **13** occurs with P–O fission, so the EREF value for phosphonylation is therefore $> 2 \times 10^6$. Clearly, the enzyme is facilitating P–N fission almost as effectively as it does C–N fission in β-lactams. Despite the differences in geometrical requirements for substitution at acyl and phosphyl centres and the enormous differences in intrinsic chemical reactivities between the β-lactam in penicillin and the phosphonamidate, the β-lactamase enzyme is able to significantly lower the activation energy for reactions of both compounds.

The generation of a formal negative charge on oxygen in the tetrahedral intermediate is accompanied by a large change in the basicity of the oxygen—a change in pK_a of the corresponding conjugate acids of at least 12 pK_a units (Scheme 6). The kinetically important species of the tetrahedral intermediate is its anion even though the thermodynamically more stable form is the neutral conjugate acid at neutral pH. Nonetheless, the anion must be strongly solvated by hydrogen-bonding and many enzymes which catalyse acyl transfer reactions have an 'oxyanion hole' which stabilises the tetrahedral intermediate by hydrogen-bonding from adjacent peptide links. The *difference* in this interaction between the initial state carbonyl oxygen and the tetrahedral intermediate as a result of the change in oxygen basicity makes a major contribution to the lowering of the activation energy compared with the non-enzyme-catalysed reaction.[54] Such an 'oxyanion hole' exists for the serine β-lactamases[55] and, in common with serine proteases, one of the peptide N–H hydrogen-bond donors is that belonging to the serine residue—Ser70 in the class A enzymes and Ser64 in the class C enzymes. The 'intramolecular' hydrogen-bond with the active site serine peptide NH presents a fairly well-defined geometry. In the equivalent pentavalent phosphyl enzyme the negative charge on the oxygen presumably takes up an equatorial position so that it is *ca*. 90° to the newly formed serine O–P bond compared with the approximately tetrahedral angle formed with the β-lactam substrate (Scheme 6). Despite these differences, the enzyme is capable of catalysing the phosphonylation with extreme efficiency.

It is often assumed that the phosphylation of serine enzymes can only occur with organophosphorus compounds with good leaving groups because these do not require proton transfer from general acid catalysts to aid departure.[56] However, the class C β-lactamase is obviously capable of displacing the proline residue from the phosphonamidate **13** despite the poor leaving group and the presumed need for protonation of the amine nitrogen.

There are two ionisations in class C β-lactamase which control hydrolytic catalytic activity (Fig. 1)—corresponding to

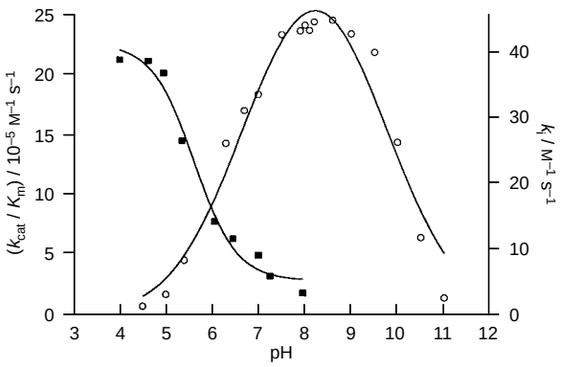
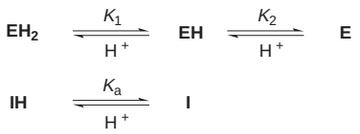


Fig. 1 (○) A plot of k_{cat}/K_m (left hand axis) for the class C *E.cloacae* β-lactamase-catalysed hydrolysis of benzylpenicillin against pH and (■) k_i (right hand axis) against pH for the inactivation of the enzyme by the phosphonamidate **13 (R = BnO)**

groups of pK_a *ca.* 6.1 and 10.1.[22] The pH-dependence of k_i (Fig. 1) shows that inactivation also depends on a catalytic group of pK_a 6.2, which suggests that the catalytic machinery used for hydrolysis is also used for the phosphonylation of **13**. However, the pH–rate profile for inactivation indicates an additional proton is required, *i.e.* whereas hydrolysis apparently requires the group of pK_a 6 to be in its deprotonated, basic form, phosphonylation apparently requires this group to be in its protonated, acidic form, which is probably the result of kinetic ambiguity. The rate of hydrolysis of the substrate **S** is proportional to [**EH**][**S**] where [**EH**] is the protonic form of the enzyme with the groups of pK_a 6 and 10 in their basic and acidic forms, respectively (Scheme 8). The observed kinetics (Fig. 1)

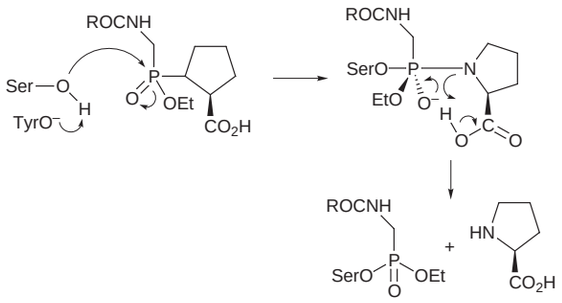


Scheme 8

for inactivation by the inhibitor **I** indicate a rate proportional to [$\mathbf{EH_2}$][**I**], which, for a process of phosphonylation of the active site serine (Scheme 7) is difficult to interpret mechanistically. Of course, the rate law can have various kinetic equivalents [eqn. (6)].

$$\text{Rate} = k_i[\mathbf{EH}_2][\mathbf{I}] = \frac{k_i}{K_1}[\mathbf{EH}][\mathbf{I}][\mathbf{H}^+] = \frac{k_i K_a}{K_1}[\mathbf{EH}][\mathbf{IH}] \quad (6)$$

Phosphonylation could occur through **EH** with the group of pK_a 6 acting as a general base for serine attack as in the hydrolytic reaction (Scheme 9). Breakdown of the trigonal bipyramidal



Scheme 9

intermediate, however, could occur by proton donation to the proline nitrogen from the solvent hydronium ion and not the protein [eqn. (6)]. Only the L-proline phosphonamidate **13** (R = BnO) is an effective inhibitor; the corresponding ester is ineffective and the D-proline isomers are much weaker. This could reflect the normal requirement of a carboxylate anion for molecular recognition. It could, however, indicate that the reaction occurs through **EH** and **IH** [eqn. (6)] where the latter is the conjugate acid of the phosphonamidate **13**, *i.e.* with the carboxylic acid group undissociated. The pK_a of this carboxylic acid is 3.86, so, over the pH range studied for inactivation, the major species present is the carboxylate anion of the phosphonamidate **13**. The pH-dependence for inactivation (Fig. 1) would then be given by eqn. (7). The true second-order rate constant for phosphonylation would then be k_iK_1/K_a =

$$k_{obs}^{inact} = k_i \cdot \frac{K_1}{K_a} \cdot \frac{H^+}{K_1 + H^+} \quad (7)$$

6.43×10^3 dm^3 mol^{-1} s^{-1}, giving an EREF of $> 3\times 10^9$. The class C β-lactamase is indeed very effective at enhancing the rate of phosphonylation. If the mechanism does involve the undissociated carboxylic acid of the phosphonamidate as 'substrate' then this is probably the result of 'substrate'-assisted catalysis, with the carboxylic acid acting as an intramolecular general acid catalyst facilitating P–N fission from the trigonal bipyramidal intermediate (Scheme 9).

Class B metallo-β-lactamases

Class B β-lactamases are metalloproteins which require zinc(II) ions for their activity. The first of these enzymes discovered was called β-lactamase II[57] and is produced by *Bacillus cereus*, which also produces two distinct class A enzymes. In 1985 there were just two species identified as producing metallo-β-lactamases. Now there are at least 20 bacterial sources of the metallo-enzyme including those found in *Pseudomonas maltophilia* (L-1), *Aeromonas hydrophila* (A2) and *Bacteroides fragilis*.[58]

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-β-lactamases.[59] The mechanism-based inactivators which have been used against the serine enzymes are generally ineffective against the Zn^{II}-dependent enzymes, and, at present, there are no clinically-useful inhibitors known. One of the main characteristics of the zinc-enzyme is its ability to catalyse the hydrolysis of nearly all β-lactams including carbapenems **3**. These β-lactamases are extremely efficient at catalysing the hydrolysis of imipenem with values of k_{cat}/K_m of *ca*. 10^6 M^{-1} s^{-1}, which is at least a 1000-fold greater than that shown by 'classical' class A β-lactamases such as TEM-1. A review of the catalytic properties of the well-characterised class B β-lactamases shows that the *A. hydrophila* enzyme exhibits the most specific substrate profile, while the other enzymes are rather broad-spectrum.[60]

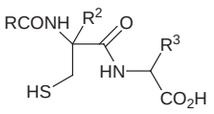
The sequences of the metallo-β-lactamases have been established and they all contain a single peptide chain, composed of 220–230 residues.[61] A sequence comparison indicates that the *P. maltophilia* enzyme is only remotely related to the others, which do appear to constitute a more homogenous group and exhibit 37 strictly conserved residues. Surprisingly, His86, one of the histidine residues which seems to be involved as Zn^{II}-binding ligands in the *Bacillus cereus* enzyme, is replaced by an Asn residue in the *A. hydrophila* β-lactamase.

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 three histidines and one cysteine.[62] A second metal-binding site was identified, but this only weakly bound Zn^{II}. 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 coordinated by three histidine residues (86, 88 and 149) and, probably, a water molecule in a distorted tetrahedral arrangement.[63] Equilibrium dialysis[64] and ^{1}H NMR analysis[65] indicate that the *B. cereus* II enzyme is capable of binding two zinc ions but computer-assisted molecular modelling indicates there is just one major metal ion binding site.[62] 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.[64] Most mechanistic and structural information is derived from the *B. cereus* enzyme. However, there has very recently been a structure reported for the binuclear zinc β-lactamase from *B. fragilis*[66] which tightly binds both Zn^{II} ions, although the loss of a single Zn^{II} is not catastrophic for β-lactamase activity.[67].

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, attacking the carbonyl group of the β-lactam ring. It was originally proposed that glutamate-37 acts as a general base and deprotonates the water molecule with the proton subsequently being donated to the nitrogen atom of the β-lactam ring to cause cleavage.[68] However, glutamate-37 is too far from the zinc ion to perform this function and site-directed mutagenesis studies have shown this glutamate is not essential for the catalytic function of the enzyme.[69] It has been demonstrated that aspartate-90 is essential for enzyme activity and consequently suggested that this residue acts as the general base to assist in the hydrolysis of the amide bond of β-lactam substrates.[63,70] 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 simply 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.

Inhibition of metallo-β-lactamases

Whatever the detailed mechanism of action of β-lactamase, it is likely that zinc(II) stabilises the tetrahedral intermediate **12** presumed to be formed during the catalytic process. Thiols are well-known inhibitors of metallo-proteases because of their ability to coordinate to the active site zinc.[34] We have synthesised several thiol derivatives with structures containing suitable sites for molecular recognition (**14**) as potential



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inhibitors of the class B metallo-β-lactamases.[71,72] The thiols are indeed competitive inhibitors of the *B.cereus* 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. The inhibition constants vary from 10 to 400 μM and are dependent on the nature of the substituents and the stereochemistry at R^2 and R^3.[71] The antihypertensive drug and ACE thiol inhibitor captopril is also an effective inhibitor with a K_i of 27 μM. Replacing the CH_2SH moiety in **14** by CO_2H gives far less effective inhibitors. As described later, these thiol inhibitors were useful in determining the pK_a of the zinc bound water.[72]

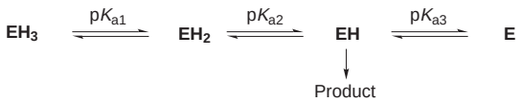
Despite the increasing clinical importance of the metallo-β-lactamases there have been few reports of effective inhibitors.[73]

Mechanism of hydrolysis

The number of binding sites for zinc(II) per molecule of class B β-lactamases appears to be one[63] or two.[66] The crystal structure of the enzyme from *B.cereus* 569/H used in our studies has one tightly bound zinc.[63] The effect of additional zinc(II) in solution on the catalytic activity of the enzyme is minimal. Between pH 5.5 and 7.0 a ten thousand-fold excess of zinc(II) increases k_{cat}/K_m by less than 36%, indicating that for this enzyme, at least, there is no catalytically important second site for zinc(II).[72]

The pH-dependence of the rate of hydrolysis of β-lactams catalysed by the class B metallo-β-lactamase from *B. cereus* is very unusual. The pH-dependence of the logarithm of the rate constants, k_{cat}/K_m and k_{cat}, for the hydrolysis of benzylpenicillin and cephaloridine show characteristic bell-shaped behaviour, but, amazingly, *the slope on the acidic limb is 2.0* and not the usual 1.0. The variations in kinetic constants are the result of reversible ionisation and are not due to acid-catalysed inactivation/degradation of the protein.[72] There is no evidence for rapid and reversible denaturation of the enzyme at pH 4.5 from changes in the CD spectrum.

It therefore appears that the rate is suppressed at lower pH because of *two* protonation processes. Because similar behaviour is observed for the two substrates, a penicillin and a cephalosporin, this unusual behaviour is attributable to protonation of the enzyme. The pK_as corresponding to these two equilibrium processes, *i.e.* pK_{a1} and pK_{a2} in Scheme 10, must be



Scheme 10

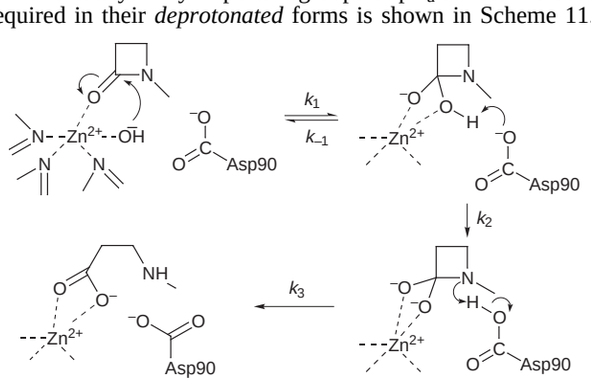
similar, with pK_{a1} = pK_{a2} = 5.60 ± 0.20. Altogether, there are *three* kinetically important ionising groups including one with a pK_a of 9.5 (Scheme 10). The mechanism may not necessarily involve the two groups of pK_a 5.6 and that of pK_a 9.5 in their deprotonated and acidic forms, respectively. The kinetics simply indicate that there are three basic residues and a proton controlling activity. Two of the most likely candidates for these ionisations are the zinc-bound water molecule and Asp90. The zinc of *B. cereus* β-lactamase is co-ordinated to three protein ligands—His86, His88 and His149—and a water molecule.[63] Formally, either the pK_a of 5.6 or 9.5 could conceivably correspond to the ionisation of zinc-bound water. The pK_a of hydrated Zn^{2+} is 9.5 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[74] and in carboxypeptidase A it is 6.2.[75] The most likely amino acid candidate for the pK_a of 5.6 is aspartate-90, which is highly conserved.

It has been possible to deduce the pK_a of the zinc-bound water in β-lactamase by determining the dependence of the inhibition constant pK_i of the thiols **14** against pH. Binding is *pH-independent* between pH 6 and 9 but decreases in both acidic and basic solution—the inflections are very similar to those observed for enzyme-catalysed hydrolysis which indicates that the ionisation states of the enzyme required for catalysis are also those required for binding the inhibitor. However, the slope on the acidic limb is 1.0, in contrast to that observed for hydrolysis, which implies that only one acidic group in the enzyme has an effect on binding the inhibitor. The pH-independent binding of the thiol inhibitor between pH 6 and 9 indicates that the pK_a of the zinc-bound water is the low value of 5.6 because the only scheme which gives pH-independent binding between pH 6 and 9 would be if the neutral undissociated thiol, pK_a 9.5, binds to the species of enzyme in which the water bound to the zinc is fully deprotonated [eqn. (8)].[73]

$$EZn{\cdot}OH + RSH \rightleftharpoons EZn{\cdot}SR + H \qquad (8)$$

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 carbonyl group 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 exists 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. Although C–N bond fission is the most energetically difficult process in amides hydrolysis, little attention is normally given to the mechanism of the breakdown of the tetrahedral intermediate.

The mechanism which is compatible with the observation that two catalytically important groups of pK_a *ca.* 5.6 are both required in their *deprotonated* forms is shown in Scheme 11.



Scheme 11

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 stabilise the negative charge generated on the carbonyl oxygen on forming the tetrahedral intermediate. Ring opening of the β-lactam ring is not a facile process and this first step 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 ($k_{-1} >> k_2$, Scheme 11). This is not surprising; 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. In the metallo-β-lactamase mechanism this process could occur by the carboxylate anion of Asp90 (Scheme 11) 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. 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 Asp90 donating a proton to the departing amine nitrogen (Scheme 11). Either k_2 or k_3 could be rate-limiting, and kinetic solvent isotope effects do not distinguish between these. The proposed mechanism also suggests structures which may be suitable for inhibiting the metallo-β-lactamases.

Summary

The battle between humans and bacteria appears relentless and the ability of bacteria to develop and pass on resistance to antibiotics seems never-ending. Understanding the mechanisms of the broad classes of β-lactamases makes one contribution to overcoming this conflict.

Although there is superficial similarity between the serine β-lactamases and the more familiar serine proteases the details of the mechanisms of the necessary proton transfer steps remains unknown. We need to know more about the mechanics of C–N bond fission in the β-lactam leading to ring opening. Given the relative conformational inflexibility of the normal β-lactam substrates it is intriguing to note the apparent flexibility of the enzyme in efficiently catalysing the reactions of a wide variety of substrates and inactivators. There does *not* appear to be an exquisite positioning of the catalytic groups and, indeed, the detailed mechanism may vary with substrate structure. This may be an advantage with respect to the design of potential enzyme inactivators and inhibitors. In addition to acyl and phosphyl transfer the serine β-lactamases may catalyse other reactions.

The metallo-β-lactamases are becoming clinically more important as a form of bacterial resistance. The mechanism of their action again appears to be different from the formally similar metallo-proteases. Now that it appears that a dianionic tetrahedral intermediate is involved, the design of suitable transition state analogues may lead to effective inhibitors of the zinc-dependent β-lactamases. The importance of a second metal ion in the metallo-β-lactamases remains unknown.

Mike Page is Dean of the School of Applied Sciences at the University of Huddersfield. He obtained his Ph.D. under the supervision of Brian Capon at the University of Glasgow in 1970 and subsequently worked with Bill Jencks at Brandeis on the energetics of intramolecular reactions and enzyme catalysis. Following a post-doctoral position with Ronnie Bell at Stirling on proton-transfer he moved to Huddersfield in 1973. His research interests are organic and bioorganic reaction mechanisms, enzyme catalysis and inhibition and the use of enzymes in synthesis. In 1991 he was a recipient of the 150th anniversary *Perkin Transactions* prize.

Andy Laws graduated from Lancaster University before moving to undertake a Ph.D. in Physical Organic Chemistry at Sussex University with Dr R. Taylor. In 1987 he took up a post-doctoral fellowship at Huddersfield University with M. I. Page. Presently he holds the post of Senior Lecturer in Organic Chemistry and still works very closely with Mike Page.

Notes and References

† E-mail: m.i.page@hud.ac.uk

1 H. C. Neu, in *The Chemistry of β-Lactam*s, ed. M. I. Page, Blackie, Glasgow, 1992, pp. 101–128.
2 M. I. Page, *Adv. Phys. Org. Chem.*, 1987, **23**, 165.
3 S. G. Waley, in *The Chemistry of β-Lactams*, ed. M. I. Page, Blackie, Glasgow, 1992, pp. 198–226.
4 D. J. Payne, *J. Med. Microbiol.*, 1993, **39**, 93; P. Nordmann, S. Mariotte, T. Naas, R. Labia and M. H. Nicolas, *Antimicrob. Agents Chemother.*, 1993, **37**, 939; T. Naas, L. Vandel, W. Sougakoff, D. M. Livermore and P. Nordmann, *Antimicrob. Agents Chemother.*, 1994, **38**, 1262.
5 K. Bush, G. A. Jacoby and A. A. Modeiros, *Antimicrob. Agents Chemother.*, 1995, **39**, 1211.
6 V. Hechler, M. Van den Weghe, H. H. Martin and J.-M. Frère, *J. Gen. Microbiol.*, 1989, **135**, 1275.
7 M. I. Page, *The Chemistry of β-Lactams*, ed. M. I. Page, Blackie, Glasgow, 1992, pp. 79–100.
8 N. J. Baxter, A. P. Laws, L. Rigoreau and M. I. Page, *J. Chem. Soc., Perkin Trans. 2*, 1996, 2245.
9 A. P. Laws, J. R. Stone and M. I. Page, *J. Chem. Soc., Chem. Commun.*, 1994, 1223.
10 P. Webster, L. Ghosez and M. I. Page, *J. Chem. Soc., Perkin Trans. 2*, 1990, 805.
11 A. M. Davis, P. Proctor and M. I. Page, *J. Chem. Soc., Perkin Trans. 2*, 1991, 1213.
12 M. I. Page, *Phil. Trans. R. Soc. Lond. Ser. B*, 1991, **332**, 149.
13 A. F. Martin, J. J. Morris and M. I. Page, *J. Chem. Soc., Chem. Commun.*, 1979, 298; N. P. Gensmantel and M. I. Page, *J. Chem. Soc., Perkin Trans. 2*, 1979, **137**; J. J. Morris and M. I. Page, *J. Chem. Soc., Perkin Trans. 2*, 1980, 212.
14 A. Llinas-Marti, B. Vilanova, J. Frau, F. Munoz, J. Donoso and M. I. Page, submitted for publication in *J. Org. Chem*.
15 J. A. Kelly, O. Dideberg, P. Charlier, J. Wery, M. Libert, P. Moews, J. Knox, C. Duez, C. Fraipont, B. Joris, J. Dusart, J.-M. Frère and J. M. Ghuysen, *Science*, 1986, **231**, 1429.
16 B. Samraoui, B. Sutton, R. Todd, P. Artmiuk, S. G. Waley and D. Phillips, *Nature*, 1986, **320**, 378.
17 J. R. Knox and P. C. Moews, *J. Mol. Biol.*, 1991, **220**, 435; P. C. Moews, J. R. Knox, O. Dideberg, P. Charlier and J.-M. Frère, *Proteins Struct. Funct. Genet.*, 1990, **7**, 156; O. Herzberg and J.-M. Moult, *Science*, 1987, **236**, 694; O. Herzberg, *J. Mol. Biol.*, 1991, **217**, 701; O. Dideberg, O. Charlier, J.-P. Wéry, P. Dehottay, J. Dusart, T. Erpicum, J.-M. Frère and J.-M. Ghuysen, *Biochem. J.*, 1987, **245**, 911; J. Lamotte-Brasseur, G. Dive, O. Dideberg, P. Charlier, J.-M. Frère and J.-M. Ghuysen, *Biochem. J.*, 1991, **279**, 213; C. Jelsch, L. Mourey, J.-M. Masson and J.-P. Samama, *Proteins Struct. Funct. Genet.*, 1993, **16**, 364.
18 C. Oefner, A. Darcy, J. J. Daly, K. Gubernator, R. L. Charnes, I. Heinze, C. Hubschwerlen and F. K. Winkler, *Nature*, 1990, **343**, 284.
19 E. Lobkovsky, P. C. Moews, L. Hansong, Z. Haiching, J.-M. Frère and J. R. Knox, *Proc. Natl. Acad. Sci. U.S.A.*, 1993, **90**, 11 257.
20 Y.-C. Leung, C. V. Robinson, R. T. Aplin and S. G. Waley, *Biochem. J.*, 1994, **299**, 671; R. P. A. Brown, R. T. Aplin and C. J. Schofield, *Biochemistry*, 1996, **35**, 12421.
21 J. Fisher, J. G. Belasco, S. Khosla and J. R. Knowles, *Biochemistry*, 1980, **19**, 2895.
22 M. I. Page, B. Vilanova and N. J. Layland, *J. Am. Chem. Soc.*, 1995, **117**, 12 092.
23 H. Christensen, M. T. Martin and S. G. Waley, *Biochem. J.*, 1990, **266**, 853.
24 N. C. J. Strynadka, H. Adachi, S. E. Jensen, K. Johns, A. Sielecki, C. Betzel, K. Sutoh and M. N. G. James, *Nature*, 1992, **359**, 700.
25 M. I. Page and A. Williams, in *Organic and Bioorganic Mechanisms*, Longman, 1997.
26 A. P. Laws and M. I. Page, *J. Chem. Soc., Perkin Trans. 2*, 1989, 1577.
27 S. C. Buckwell, M. I. Page and J. L. Longridge, *J. Chem. Soc., Perkin Trans. 2*, 1988, 1809; S. C. Buckwell, M. I. Page, S. G. Waley and J. L. Longridge, *J. Chem. Soc., Perkin Trans. 2*, 1988, 1815; S. C. Buckwell, M. I. Page, J. L. Longridge and S. G. Waley, *J. Chem. Soc., Perkin Trans. 2*, 1988, 1823; A. P. Laws, N. J. Layland, D. G. Proctor and M.

I. Page, *J. Chem. Soc., Perkin Trans. 2*, 1993, 17; N. J. Layland, A. P. Laws, B. Vilanova and M. I. Page, *J. Chem. Soc., Perkin Trans. 2*, 1995, 869.
28 A. Matagne and J.-M. Frère, *Biochem. Biophys. Acta*, 1995, **1246**, 109.
29 M. I. Page, *Chem. Soc. Rev*., 1973, **2**, 295; M. I. Page and W. P. Jencks, *Gazz. Chim. Ital*., 1987, **117**, 455.
30 C. V. Barker and M. I. Page, unpublished results.
31 K. Miyashita, I. Massova, P. Taibi and S. Mobashery, *J. Am. Chem. Soc*., 1995, **117**, 11 055; I. Massova and S. Mobashery, *Acc. Chem. Res.*, 1997, **30**, 162.
32 A. Matagne, J. Lamotte-Brasseur, G. Dive, J. R. Knox and J.-M. Frère, *Biochem. J*., 1993, **293**, 607.
33 J. Rahil and R. F. Pratt, *Biochemistry*, 1994, **33**, 116.
34 M. I. Page, in *Comprehensive Medicinal Chemistry*, ed. P. G. Sammes, Pergamon, Oxford, 1990, vol. 2, pp. 61–87.
35 W. J. Albery and J. R. Knowles, *Biochemistry*, 1976, **15**, 5631; M. I. Page, in *The Chemistry of Enzyme Action*, ed. M. I. Page, Elsevier, Oxford, 1984 pp. 1–54; M. I. Page, in *Comprehensive Medicinal Chemistry*, ed. P. G. Sammes, Pergamon, Oxford, 1990, vol. 2, pp. 45–60.
36 H. Christensen, M. T. Martin and S. G. Waley, *Biochem. J.*, 1990, **266**, 853.
37 O. Herzberg and J. Moult, *Curr. Opin. Struct. Biol.*, 1991, **1**, 946; N. C. J. Strynadka, R. Martin, S. E. Jensen, M. Gold and J. B. Jones, *Nature Struct. Biol.*, 1996, **3**, 688.
38 C. Damblon, X. Raquet, L. Lu-Yun, J. Lamotte-Brasseur, E. Fonzé, P. Charlier, G. C. K. Roberts and J.-M. Frère, *Proc. Natl. Acad. Sci. U.S.A.*, 1996, **93**, 1747.
39 X. Raquet, V. Lounnas, J. Lamotte-Brassuer, J.-M. Frère and R. C. Wade, *Biophys. J.*, 1997, **73**, 2416.
40 M. G. P. Page, *Biochem J.*, 1993, **295**, 295; A. Samuni and N. Citri, *Biochem. Biophys. Res. Commun.*, 1975, **62**, 7; P. Taibi-Tronche, I. Massova, S. B. Vakulenko, S. A. Lerner and S. Mobashery, *J. Am. Chem. Soc.*, 1996, **118**, 7441.
41 M. Galleni, G. Amicosante and J.-M. Frère, *Biochem. J*., 1988, **255**, 123.
42 C. Damblon, G. H. Zao, M. Jamin, P. Ledent, A. Dubus, M. Vanhove, X. Raquet, L. Christiaens and J.-M. Frère, *Biochem. J.*, 1995, **309**, 431; Y. Xu, G. Soto, K. R. Hirsch and R. F. Pratt, *Biochemistry*, 1996, **35**, 3595; B. P. Murphy and R. F. Pratt, *Biochemistry*, 1991, **30**, 3640.
43 E. Lobovsky, E. M. Billings, P. C. Moews, J. Rahil, R. Pratt and J. R. Knox, *Biochemistry*, 1994, **33**, 6762.
44 A. Bulychev, I. Massova, K. Miyashita and S. Mobashery, *J. Am. Chem. Soc*, 1997, **33**, 7620.
45 A. Dubus, S. Normark, M. Kania and M. G. P. Page, *Biochemistry*, 1994, **33**, 8577.
46 K. B. Schowen and R. L. Schowen, *Methods Enzymol.*, 1982, **87**, 551; D. M. Quinn and L. D. Sutton, *Enzyme Mechanisms from Isotope Effects*, ed. P. F. Cooke, CRC Press, 1991, pp. 73–126; A. J. Kresge, R. A. More O'Ferrall and M. F. Powell, *Isotopes in Organic Chemistry*, ed. E. Buncel and C. C. Lee, Elsevier, Amsterdam, 1987, pp. 177–273.
47 Y. Chiang, A. J. Kresge and R. A. More O'Ferrall, *J. Chem. Soc., Perkin Trans. 2*, 1980, 1832; F. Hibbert and H. J. Robbins, *J. Chem. Soc., Chem. Commun.*, 1980, 141.
48 H. B. Burgi and J. D. Dunitz, *Acc. Chem. Res.*, 1983, **16**, 153.
49 G. R. J. Thatcher and R. Kluger, *Adv. Phys. Org. Chem.*, 1989, **25**, 99.
50 F. M. Menger, *Acc. Chem. Res.*, 1985, **18**, 128; F. M. Menger, *Acc. Chem. Res.*, 1993, **26**, 206; A. Daffron and D. E. Koshland, *Proc. Natl. Acad. Sci. U.S.A.*, 1971, **68**, 2463; A. D. Mesecar, B. L. Stoddard and D. E. Koshland, Jr., *Science*, 1997, **277**, 202; A. J. Kirby, *Acc. Chem. Res.*, 1997, **30**, 290; A. J. Kirby, *Angew. Chem., Int. Ed. Engl.*, 1996, **35**, 707.
51 M. I. Page, A. P. Laws, M. J. Slater and J. R. Stone, *Pure Appl. Chem.*, 1995, **67**, 711; A. P. Laws, M. I. Page and M. J. Slater, *Bioorg. Med. Chem. Lett.*, 1993, **3**, 2317.
52 M. Jones and M. I. Page, *Chem. Commun.*, 1991, 316.
53 M. I. Page, *J. Mol. Catal.*, 1988, **47**, 241.
54 See however: W. L. Mock and D. C. Y. Chua, *J. Chem. Soc., Perkin Trans 2*., 1995, 2069.
55 B. P. Murphy and R. F. Pratt, *Biochem. J*., 1988, **256**, 669.
56 I. M. Kovach, *J. Enzyme Inhibition*, 1988, **2**, 199; I. M. Kovach and E. J. Enyedy, *J. Am. Chem. Soc.*, 1998, **120**, 258.
57 S. Kubawara and E. P. Abraham, *Biochem. J*., 1967, **103**, 23C.
58 Y. Saino, F. Kobayashi, M. Inoue and S. Mitsuhashi, *Antimocrob. Agents Chemother*., 1982, **34**, 1590; R. Bicknell, E. L. Emmanuel, J. Gagnon and S. G. Waley, *Biochem. J*., 1985, **229**, 791; J. P. Iaconis and C. C. Sanders, *Antimicrob. Agents Chemother*., 1990, **34**, 44; G. J. Cuchural, Jr., M. H. Malamy and F. P. Tally, *Antimicrob. Agents Chemother*., 1986, **30**, 645.
59 D. J. Payne, *J. Med. Microbiol.*, 1993, **39**, 93.
60 A. Felici, G. Amicosante, A. Oratore, R. Strom, P. Ledent, B. Joris, L. Fanuel and J.-M. Frère, *Biochem. J*., 1993, **291**, 151.
61 H. M. Lim, J. J. Pène and R. Shaw, *J. Bacteriol*., 1988, **170**, 2873; J.-M. Frère. *Mol. Microbiol.*, 1995, **16**, 385; O. Massidda, G. M. Rossolini and G. Salta, *J. Bacteriol.*, 1991, **173**, 4611; B. A. Rasmussen, Y. Gluzman and F. P. Tally, *Antimicrob. Agents Chemother*., 1990, **34**, 1590; J. S. Thompson and M. H. Malamy, *J. Bacteriol.*, 1990, **172**, 2584; M. Hussain, A. Carlino, M. J. Madonnam and J. O. Lampen, *J. Bacteriol.*, 1985, **164**, 223; R. P. Ambler, M. Daniel, J. Fleming, J.-M. Hermoso, C. Pang and S. G. Waley, *FEBS Lett.*, 1986, **189**, 207.
62 B. J. Sutton, P. J. Artymiuk, A. E. Cordero-Borboa, C. Little, D. C. Phillips and S. G. Waley, *Biochem. J.*, 1987, **248**, 181.
63 A. Carfi, S. Pares, U. Duée, M. Galleni, C. Duez, J.-M. Frère and O. Dideberg, *EMBO J*., 1995, **14**, 4914.
64 R. B. Davies and E. P. Abraham, *Biochem. J*., 1974, **143**, 129.
65 G. S. Baldwin, A. Galdes, H. A. O. Hill, B. E. Smith, S. G. Waley and E. P. Abraham, *Biochem. J*., 1978, **175**, 441.
66 N. O. Concha, B. A. Rasmussen, K. Bush and O. Herzberg, *Structure*, 1996, **4**, 823; *Protein Sci.*, 1997, **6**, 2671; A. Carfi, R. Paul-Soto, L. Martin, Y. Petillot, J.-M. Frère and O. Dideberg, *Acta. Crystallogr., Sect. D*, 1997, **53**, 485.
67 M. W. Crowder; Z. Wang, S. C. Franklin, E. P. Zovinka and S. J. Benkovic, *Biochemistry*, 1996, **35**, 12126.
68 C. Little, E. L. Emanuel, J. Gagnon and S. G. Waley, *Biochem. J*., 1986, **233**, 465.
69 H. M. Lim and J. J. Pène, *J. Biol. Chem.*, 1989, **264**, 11 682.
70 H. M. Lim, R. K. Iyer and J. J. Pène, *Biochem. J*, 1991, **276**, 401.
71 S. Bounaga and M. I. Page, unpublished results.
72 S. Bounaga, A. P. Laws, M. Galleni and M. I. Page, *Biochem. J*., 1998, **331.**
73 M. W. Walker, A. Felici, M. Galleni, R. P. Soto, R. M. Adlington, J. E. Baldwin, J.-M. Frère, M. Gololobov and C. J. Schofield, *Bioorg. Med. Chem Lett.*, 1996, **6**, 2455; D. J. Payne, J. H. Bateson, B. C. Gasson, D. Proctor, T. Khushi, T. H. Farmer, D. A. Tolson, D. Bell, P. W. Skett, A. C. Marshall, R. Reid, L. Ghosez, Y. Combret and J. Marchand-Brynaert, *Antimicrob. Agents Chemother*., 1997, **41**, 135; J. T. Toney, P. M. D. Fitzgerald, N. Grover-Sharma, S. H. Olsen, W. J. May, J. G. Sundelot, D. E. Vanderwall, K. A. Cleary, S. K. Grant, J. K. Wu, J. W. Kozarich, D. L. Pompliano and G. H. Hammond, *Chem. Biol.*, 1998, **5**, 185.
74 Y. Pocker and D. W. Bjorkquist, *Biochemistry*, 1977, **16**, 5698; E. Kimura, T. Shiota, T. Koike, M. Shiro and M. Kodama, *J. Am. Chem. Soc.*, 1990, **112**, 5805.
75 W. L. Mock and J.-T. Tsay, *Biochemistry*, 1986, **25**, 2920; W. L. Mock and J.-T. Tsay, *J. Biol. Chem.*, 1988, **263**, 8635.

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