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THE MECHANISM OF REACTIONS CATALYSED BY THE SERINE β-LACTAMASES

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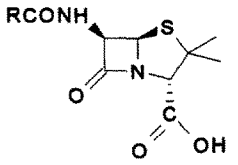
Abstract: Nucleophilic substitution at the β-lactam carbonyl of penicillins usually requires general base and acid catalysis and proceeds via the formation of a tetrahedral intermediate. A class C serine β-lactamase is inhibited by phosphonamidates, shows diastereoselectivity in P-N and P-O bond fission and exhibits enzyme catalysed nucleophilic displacement of proline. The requirements for general acid catalysed expulsion of proline have interesting stereochemical consequences.

Penicillin **(1)** was the first naturally occurring antibiotic to be characterised and used in clinical medicine. It is now seen as the progenitor of the β lactam family of antibiotics which are characterised by the possession of the four membered ring.[1] Together with the cephalosporins **(2)**, the penicillins are the most commonly used antibiotics. However many bacteria are resistant to the normally lethal action of these compounds



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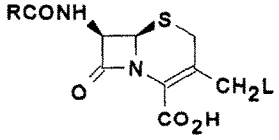

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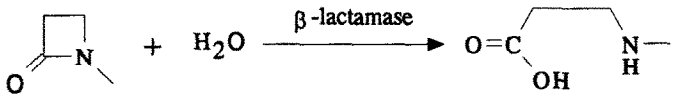
mainly because of their ability to produce a β-lactamase enzyme which catalyses the hydrolysis of the β-lactam to its corresponding amino acid (Scheme 1), devoid of antibacterial activity. There are two main classes of β-lactamases - the serine and the

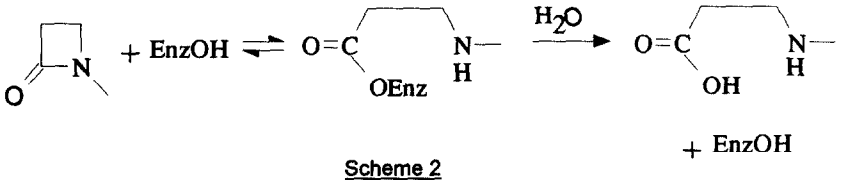


Scheme 1

zinc enzymes.[2] The former contain an active site serine and are characterised by the intermediate formation of an acyl-enzyme (Scheme 2).

Investigating the efficiency and mechanism of enzyme catalysis often involves determining the effect on reactivity of changing substituents in the substrate or the enzyme and the reaction conditions such as the nature of the solvent. Changes in the substrate structure may modify its intrinsic chemical reactivity in terms of inductive, resonance and steric effects on the ease of bond making and breaking, in addition to

modifying its binding interactions with the enzyme and hence may have a complex effect on catalytic activity. Changes in the enzyme structure may not only modify the binding and catalytic interactions but also the conformation of the enzyme Changes in

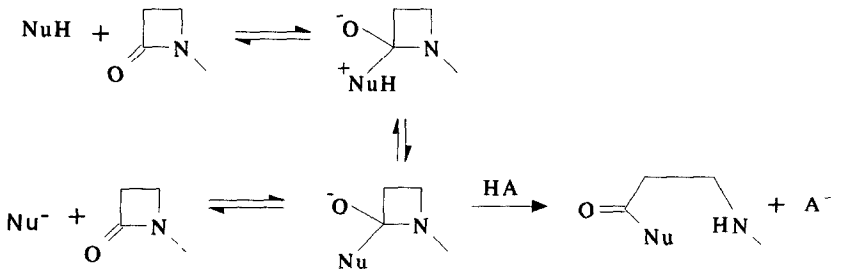


Scheme 2

solvent may affect the ground state energies of the enzyme and the substrate as well as the transition state energy.[3] Reactivity, as measured by rate constants, reflects the difference in energies between the ground state and transition state. In order to deduce the importance and role of interactions between the enzyme and substrate it is necessary to separate the intrinsic effects to give an enzyme rate enhancement factor so that those effects resulting from specific interactions can be identified.[4]

Intrinsic Reactivity

The susceptibility of β-lactams to ring opening is markedly influenced by substituent effects[5] which can, for example, alter the reactivity towards hydroxide in catalysed hydrolysis by up to a factor of 10^6. Electron withdrawing groups attached to the amine leaving group have a particularly activating effect.[5]



Scheme 3.

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 3). In these types of reactions (except

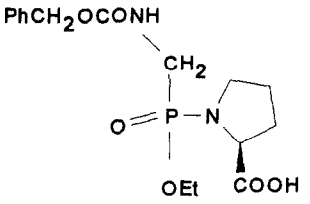
for some in very strong acid) the mechanism always involves a two step process.[1,6] Covalent bond formation to the incoming nucleophile occurs before C-N bond fission resulting in the reversible formation of a tetrahedral intermediate. Contrary to expectations, opening the four membered ring is not a facile process.[7] 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.[6]
Those reactions which involve the attack of a neutral nucleophile with an ionisable hydrogen (Scheme 3) invariably require general base catalysis to remove the proton.[5,6] The requirement for proton removal is paramount - and in extreme cases only the reaction of the anionic nucleophile is observed (Scheme 3). For example, there is no pH independent reaction of water with penicillin and alcohols react only through their anions.[8] The importance of general base catalysis is a reflection of the fact that, again contrary to expectations, penicillins are not powerful acylating agents.[8]
Similarly, C-N bond fission requires protonation of the amine nitrogen.[5,6] Amine anion expulsion from the tetrahedral intermediate is an unlikely process even considering the release of strain energy facilitating ring opening and C-N bond fission. Consequently, general acid catalysed breakdown of the tetrahedral intermediate is often observed.
It has been suggested that ring opening does not occur by stretching of the C-N bond but rather by a rotational motion.[3,7] This will minimise strain effects and maximise 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.

β-Lactamase Structure

The class A β-lactamases are produced by both Gram-positive and Gram-negative bacteria. Crystal structures of the Gram-positive β-lactamases from *Staphylococcus aureus*[9] and from *Bacillus licheniformis*[10] have been reported at 2.0Å resolution. The crystal structure of a Gram-negative β-lactamase, RTEM-1, from *Escherichia coli* at 2.5Å resolution has been published.[11] The crystal structure of the class C β-lactamase from *Citrobacter freundii* has been refined at 2.0Å resolution.[12] There have been no structures reported of good inhibitors bound to β-lactamase, although there has been a recent publication of the structure of an acyl-enzyme of a mutant RTEM-1 β-lactamase (glu 166 asn) which catalyses the acylation but not the deacylation reaction.[13] So, although useful, mechanistic conclusions based on crystal structures remain speculative.

Although the different β-lactamase have topographically similar crystal structures there are significant differences in the relative orientations of secondary structural units and conformations of some of the loop regions. There are two closely packed domains and the polypeptide chain crosses twice from one domain to the other. The active site serine 70 is located at the interface between the domains and at the amino terminus of a largely desolvated helix. Within the active site region surrounding the catalytically active serine 70 there is an invariant lysine residue (73 in class A) one helical turn after the serine. The serine oxygen to lysine nitrogen distance varies between 2.5 and 2.8 Å Another potential catalytic group within the active site is glu 166. The glu-166 carboxylate oxygen-lysine 73 nitrogen distance is 2.8 to 3.4 Å whereas the glu-166 oxygen and ser-70 oxygen are 3.5 to 4.0 Å apart. Glu-166 is apparently held pointing into the active site by the adjacent peptide being in the cis conformation.[10]

By analogy with the mechanism of acylation reactions of ß-lactams involving ring opening, the ß-lactamase catalysed reaction probably also involves the formation of a tetrahedral intermediate (Scheme 3). Although the acyl transfer process requires a number of steps involving proton transfer, little is known about the mechanism of these in the enzyme reaction and any consequent requirements for general acid - base catalysis.[2]



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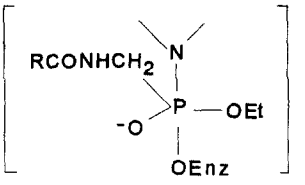

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Inactivation by Phosphonamidates

Four co-ordinate tetrahedral phosphorus derivatives are known to be inhibitors of serine enzymes either by acting as phosphorylating agents or as transition state analogues of the tetrahedral intermediate.[14] The phosphonamidates **(3)** are readily prepared using known procedures[15] and exist as a pair of diastereoisomers which may be separated by HPLC using a Dynamax 60 A reverse phase C18 column (25cm x 2.14cm, flow rate 12ml/min) and eluting with 13% acetonitrile - 87% (v/v) of an aqueous ammonium acetate solution (1% w/v). Both diastereoisomers completely and irreversibly inactivate the class C ß-lactamase (P99) from *Enterobacter cloacae* in a time dependent manner. The pseudo first-order rate constants for inactivation (k_{inact}) were determined by measuring the loss of activity of the ß-lactamase catalysed hydrolysis of benzylpenicillin **(1)** at various initial phosphonamidate concentrations.

Saturation kinetics were not observed and the overall second order rate constants for inactivation were obtained from the dependence of k_{inact} on phosphonamidate concentration. The second order rate constants measured for the individual diastereoisomers at pH 7.0 and at 30°C were 5.10 $M^{-1}s^{-1}$ and 0.14 $M^{-1}s^{-1}$. The enzyme thus displays a selectivity of 36 fold between the two diastereoisomers.

The time dependent inactivation of ß-lactamase is indicative, but not proof, of covalent bond formation between the enzyme and the phosphonamidates. A comparison with the inhibition of other enzymes by phosphorus derivatives[14,16] suggests that this could be due to displacement of either ethanol or proline from the phosphonamidate (**3**) by the active site serine. In the absence of enzyme, the phosphonamidate (**3**) undergoes predominantly P-N cleavage in acid solution but P-O fission at high pH. There is no evidence for ethanol formation during the inactivation of ß-lactamase by the more active diastereoisomer. Electrospray mass spectroscopy (ESMS) indicates, but again does not prove, that the more active diastereoisomer becomes covalently linked to the enzyme during the inactivation. The positive ion ESMS mass transformed spectrum of the (P99) ß-lactamase showed a molecular mass Mr = 39,202 ± 5.3. After incubation with the more reactive phosphonamidate (**3**) the mass spectrum gave Mr = 39,442 ± 3.1 suggesting that the phosphonylated enzyme had lost the proline residue (calculated Mr = 39,456). Inactivation thus appears to be accompanied by the formation of a covalently bound 1:1 enzyme:inactivator complex in which a proline residue has been displaced by a nucleophilic group on the enzyme - presumably the active site serine.

Direct displacement of the extremely poor leaving group the proline anion, via an $S_N2(P)$ mechanism is highly unlikely.[18] Protonation of the phosphonamidate nitrogen is almost certainly required to expel neutral proline. However, the pK_a of the protonated phosphonamidate nitrogen is less than zero so that the concentration of the protonated species present at pH 7 is so small as to be kinetically insignificant. The observed loss of proline can be accounted for if a general acid is present in the enzyme's active site and which is in a position to be able to protonate the nitrogen once the trigonal bipyramidal intermediate has been formed.

In the absence of the enzyme, the phosphonamidates (**3**) are stable in water at pH 7 under the conditions and timescale required for inactivation. The first order rate constant for the hydrolysis of (**3**) in the absence of the enzyme is 2 x $10^{-7}s^{-1}$ and the second order rate constant for the hydroxide-ion catalysed hydrolysis is 2 x 10^{-5} $M^{-1}s^{-1}$. The latter reaction involves P-O bond fission so the enzyme rate enhancement factor for P-N fission is at least 2 x 10^6. Effective enzyme catalysed phosphonylation is surprising in view of the presumed different geometries for the reaction (trigonal bipyramidal (**4**)) compared with that for acylation (tetrahedral).[16] It is often assumed[16,17] that phosphonylating agents of serine enzymes usually require good

leaving groups because these different geometries preclude general acid catalysed expulsion from the phosphorus apical position[18] by the enzyme's general acid used for acylation reactions. If expulsion of proline occurred from the equatorial position then the geometries for acylation and phosphonylation would be similar.

During the inactivation of the (P99) ß-lactamase by the less reactive diastereoisomer ethanol is displaced. Incubation of this diastereoisomer (5×10^{-4} M) with the enzyme (1×10^{-4} M) gives a time dependent release of ethanol detectable by gas chromatography (BP20 capillary column of dimensions 20M x 2.3mm).

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