pH Dependence of and Kinetic Solvent Isotope Effects on the Methanolysis and Hydrolysis of β-Lactams Catalyzed by Class C β-Lactamase

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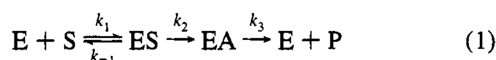
Abstract: The methanolysis of benzylpenicillin is catalyzed by *Enterobacter cloacae P99* class C β-lactamase and the pH dependence of k_{cat} indicates that the catalytic groups involved of pK_a ca. 5 and 10 are the same as those for hydrolysis. The kinetic solvent isotope effect (KSIE) $k_{cat}^{H_2O}/k_{cat}^{D_2O}$ is 1.42 for both the hydrolysis and methanolysis of benzylpenicillin. However, there is an inverse KSIE on k_{cat}/K_m of 0.83 ± 0.05 for the hydrolysis of benzylpenicillin and cephaloridine. There is also an abnormally high shift in the low pK_a on going from H_2O to D_2O of 0.85 ± 0.15 from the pH dependence of both k_{cat} and k_{cat}/K_m for both methanolysis and hydrolysis. The D_2O shift on the high pK_a of ca. 10 is the normal value of ca. 0.4. These results are consistent with a strongly hydrogen bonded system acting as the general base catalyst and low fractionation factors for the hydrogens involved. It is probable that this represents the tyrosine phenoxide ion hydrogen bonded to two lysine ammonium ions.

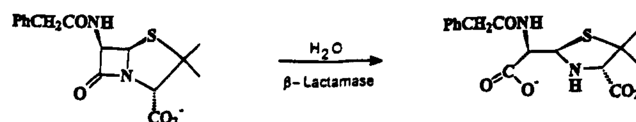
The major cause of the resistance of some bacteria to the normally lethal action of β-lactam antibiotics is the ability of the bacteria to produce β-lactamase enzymes, which catalyze the hydrolysis of the β-lactam of penicillins **1** (Scheme 1) and cephalosporins. The most prevalent and clinically important β-lactamases are the class A and C type which are both serine enzymes and act by a mechanism involving the formation of a relatively unstable acyl enzyme (EA) intermediate, eq 1.[1]



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

Although there have been several crystal structures of β-lactamases and their derivatives reported[2,3,4] and the nature and degree of the conservation of the amino acid residues in and near the active site are known,[5] there is little detailed evidence of the groups presumed to be involved with the necessary proton transfer steps.[6,7] Comparison with serine proteases would indicate the need for general acid–base catalysis.[8] The main contenders for these roles are, in class A

Scheme 1



β-lactamase, the carboxyl group of Glu-166 and the amino group of Lys-73. Interestingly, there is a *cis* peptide between Glu-166 and residue 167. In class C β-lactamase there is no equivalent glutamate residue, but tyrosine-150 may take its role with Lys-67 equivalent to Lys-73 in the class A enzyme.[2]

The second-order rate constant k_{cat}/K_m–pH profiles for the hydrolysis of β-lactams catalyzed by the serine β-lactamases have a characteristic bell shape. Although, in the simplest schemes, such profiles would indicate the important ionizing residues involved in catalysis in the *free* enzyme and substrate for the acylation step only, they do vary slightly with substrate.[9] The ionization in the acidic limb of the pH–rate profile is about 5 in class A β-lactamase but about 6 in the class C enzyme. It is generally assumed that this pK_a corresponds to that of the general base catalyst used in formation of the tetrahedral intermediate for acylation. It has been suggested that for class A β-lactamase this could be Lys-73 with a largely reduced pK_a[4] or Glu-166, although there have been conflicting reports on the role of the carboxylate anion based on site-directed mutagenesis. Some studies have suggested that the glutamate residue is equally important in both acylation and deacylation,[10,11] whereas others have indicated an important role in deacylation only but not in acylation.[12,13] In class C β-lactamase it has been

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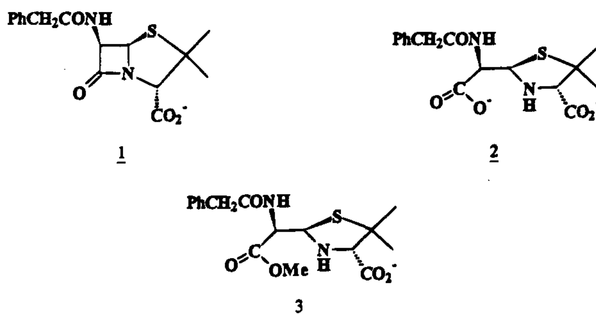
suggested that the phenol of tyrosine 150 has a severely reduced pK_a and acts as a general base catalyst for proton removal from serine 64[2,14] although this is not supported by site-directed mutagenesis of Tyr-150.[15]

The *Amp C* and *Pseudomona aeruginosa* class C β-lactamase catalyzed hydrolysis of benzylpenicilin has been shown to yield, in addition to the normal hydrolysis product, the penicilloyl α-methyl ester in the presence of methanol.[16] This is presumably due to the partitioning of the acylenzyme intermediate between its reaction with water to give the hydrolysis product and its reaction with methanol to produce the penicilloyl ester. If the methanolysis reaction is catalyzed by the enzyme, it is of interest to compare the catalytic apparatus used with that for hydrolysis. We have therefore measured the pH dependence of the rate of enzyme-catalyzed methanolysis and hydrolysis reactions and determined the solvent isotope effects on the apparent pK_as of the catalytic groups and the kinetic parameters.

Experimental Section

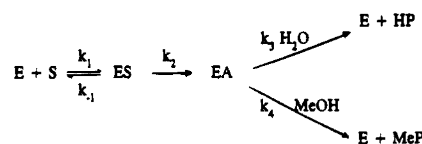
Methyl (3*S*,5*R*,6*R*)-Benzylpenicilloate (3) was prepared as previously described,[17] δ_H (D_2O) 1.22 (s, 3H, 2-α-Me), 1.52 (s, 3H, 2-β-Me), 3.59 (s, 1H, 3-H), 3.79 (s, 2H, PhCH_2), 3.84 (s, 3H, OMe), 4.70 (d, 1H, 6-H, $J_{6,5}$ = 6 Hz), 5.20 (d, 1H, 5-H, $J_{5,6}$ = 6 Hz), and 7.37 (s, 5H, Ph). HPLC (using an 8μm polystyrene−divinylbenzene polymer reversed phase column and eluting with a 30% (v/v) acetonitrile−70% aqueous solution of 0.1 M phosphoric acid containing 4.8 × 10^{-3} M hexane sulfonic acid) retention times: benzylpenicilloate (**2**), 4.6 min; methyl benzylpenicilloate (**3**), 8.0 min; and benzylpenicillin (**1**), 10.4 min.



Enterobacter cloacae class C β-lactamase was obtained from the Centre for Applied Microbiology and Research, Porton Down, UK. This enzyme has been extensively studied and its physicochemical properties and kinetic parameters for various substrates well established.[5] All kinetic experiments with the enzyme used, as a standard control, the kinetic parameters for the hydrolysis of cephaloridine at pH 7.0 and 30 °C.

Kinetic Measurements. All experiments were performed at 30.0 °C with buffers maintained at ionic strength 1.0 M with KCl. The buffers used were acetate (pH 4.0−5.8), MOPS (pH 6.0−8.0), TAPS (pH 8.0−9.3), and bicarbonate (pH 9.4−11.0). The concentration of enzyme used was 0.2−0.8 μM, and that of the β-lactam antibiotic 0.4−0.8 mM. The kinetic parameters were determined spectrophotometrically at 235 nm for benzylpenicillin and at 260 nm for cephaloridine and calculated as previously described.[18]

Scheme 2



Results and Discussion

In the absence of β-lactamase, the degradation of benzylpenicillin in aqueous solution containing up to 1 M methanol gives only the hydrolysis product. There is no detectable formation of the methyl ester resulting from methanol attack on the β-lactam.[19] However, in the presence of *Enterobacter cloacae P99* class C β-lactamase the methyl ester **3** is formed as shown by NMR and HPLC analysis. At pH 8, with 1 M methanol, up to 30% of the product is the methyl ester and the ratio of methanolysis to hydrolysis is independent of the concentration of the enzyme. Methyl benzylpenicilloate is stable under the reaction conditions[17,19] used to determine the kinetic parameters. The pathway given in eq 1 gives rise to eq 2 for the observed second-order rate constant k_{cat}/K_m which varies with pH according to eq (3). The variation of k_2, the acylation

$$\frac{k_{cat}}{K_m} = \frac{k_1 k_2}{k_{-1} + k_2} \tag{2}$$

$$\left(\frac{k_{cat}}{K_m}\right)_{obs} = \frac{k_1 k_2}{k_{-1} + k_2} \cdot \frac{K_a}{K_a + [H^+]} \tag{3}$$

step, and k_{-1}, the dissociation of the enzyme−substrate complex, with pH may be different which often makes the apparent pK_as derived from k_{cat}/K_m−pH profiles difficult to interpret. If k_{-1} and k_2 are of a similar magnitude the apparent pK_a may vary with substrate. A common assumption about the acyl enzyme mechanism is that the value of the second-order rate constant, k_{cat}/K_m, always reflects the process of acylation (eq 2), even if the rate-limiting step under the experimental conditions of substrate *saturation* is the deacylation step, k_3. This is true if the formation of the acyl enzyme is irreversible or, effectively, if the rate constant for deacylation, k_3, is much greater than that for the reverse step, k_{-2} (not shown). For the serine proteases, k_{-2} represents the "intermolecular" aminolysis of the acyl enzyme to regenerate the peptide. For the hydrolysis of β-lactams catalyzed by β-lactamases this step is an "intramolecular" aminolysis to regenerate the enzyme-(β-lactam) substrate complex. Although the formation of a strained four-membered β-lactam may have a significant activation energy it is not inconceivable that this intramolecular step is competitive with intermolecular hydrolysis of the acyl enzyme.[20] The interpretation of the macroscopic rate constant k_{cat}/K_m may thus often be ambiguous because its constituent microscopic rate constants extend only to the first irreversible step. The advantage of measuring the pH dependence of the deacylation step, k_3, is that this ambiguity is removed.

The methanolysis and hydrolysis reactions reported here are presumably due to a competition between methanol and water in reacting with the acyl−enzyme (EA) intermediate (Scheme 2). Under saturation conditions the pseudo-first-order rate constant, k_{cat}, is independent of substrate concentration and is

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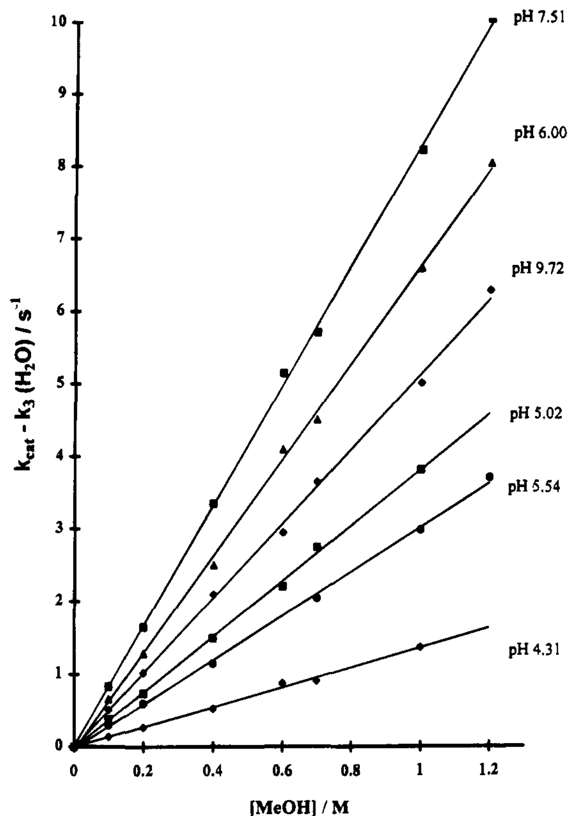

Figure 1. The dependence of $(k_{cat} - k_3)$ s^{-1} against the concentration of methanol at the indicated pH for the reaction of benzylpenicillin catalyzed by *E. cloacae P99* class C β-lactamase at 30 °C.

given by eq 4. In the absence of methanol with benzylpenicillin

$$k_{cat} = \frac{k_2(k_3 + k_4(\mathrm{MeOH}))}{k_2 + k_3 + k_4(\mathrm{MeOH})} \quad (4)$$

as the substrate hydrolyzed, k_{cat} represents mainly the deacylation rate constant k_3.[1] Using *E. cloacae* P99 class C β-lactamase with benzylpenicillin and cephaloridine as substrates in aqueous solution and in the presence of varying concentrations of methanol there is a linear dependence of k_{cat} upon the alcohol concentration (Figure 1). Under these conditions $k_2 > (k_3 + k_4(\mathrm{MeOH}))$ and k_{cat} reduces to eq 5,from which the second-

$$k_{cat} = k_3 + k_4(\mathrm{MeOH}) \quad (5)$$

order rate constant k_4 can be deduced. The kinetic parameters k_{cat}/K_m, k_{cat}, k_3, and k_4 were determined as a function of pH (pD) in H_2O and D_2O and are shown in Table 1. All rate constants show a bell-shape dependence on pH (pD) (Figure 2) indicating the importance of at least two ionizations and the apparent pK_as deduced from eq 6 are given in Table 2.

$$k_{obs} = \frac{k_{max}}{[\mathrm{H}^+]/K_a^1 + 1 + K_a^2/[\mathrm{H}^+]} \quad (6)$$

There are several points of interest arising from the kinetic data. The observation of two apparent pK_as for the methanolysis reaction and their similarity to the values observed for hydrolysis imply that the same catalytically important groups are being used for both reactions.

The kinetic solvent isotope effect (KSIE) $k_{cat}^{H_2O}/k_{cat}^{D_2O}$ is 1.42 and is the same for both methanolysis and hydrolysis which is compatible with general base catalysis. It is interesting that methanol can presumably displace water at the active site and be subjected to general base catalysis by an amino acid residue on the protein. However, there is no evidence of saturation by methanol up to 1.2 M (Figure 1) and the apparent pK_as probably reflect those of the acyl enzyme in the absence of methanol.

The ratio of the rate constants k_3/k_4 for hydrolysis to methanolysis of benzylpenicillin is 3.4 $\pm$ 0.2 and is independent of pH. It is difficult to compare these rate constants directly as k_4 is the true second-order rate constant, whereas that for k_3 is the apparent pseudo-first-order rate constant. There is some evidence[5] that there is a tightly bound water molecule used for deacylation of the acyl enzyme but taking the bulk concentration of water to convert k_3 to a second-order rate constant would indicate that methanol is the better nucleophile for the enzyme-catalyzed reaction. There is negligible enzyme-catalyzed ethanolysis although 1,2-ethanediol does increase k_{cat} and the deduced value of k_4 at pH 7.0 is 1.1 M^{-1} s^{-1}. Interestingly, 2,2,2-trifluoroethanol causes significant inhibition of hydrolysis. For example, at pH 7.0 k_{cat} is reduced by 50% in the presence of 1 M trifluoroethanol. Similarly, there was no aminolysis detected with ammonia or hydrazine. This selectivity is also enzyme specific as class A β-lactamase shows no methanolysis of benzylpenicillin.

The absolute values of 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 and 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.[21] However, ΔpK_a^1 is 0.85 $\pm$ 0.15 from k_{cat}/K_m and k_{cat} for both methanolysis and hydrolysis. This shift is outside the normal range and is indicative of a system with an unusually low fractionation factor for the basic species formed and/or a high fractionation factor for the protonic state undergoing dissociation.[22] The most likely basic group responsible for the acid limb of the pH$-$rate profile for both acylation and deacylation is Tyr-150 which is hydrogen bonded to Lys-315 and Lys-67[2,14] (Chart 1).

Low fractionation factors for the base formed upon ionization are compatible with this unusual tyrosine base with the phenoxide anion, strongly stabilized by the adjacent positively charged lysines accounting for the low pK_a, acting as a proton acceptor for both acylation and deacylation. Strong solvation of the phenoxide ion by the NH_3^+ groups is consistent with low fractionation factors for the hydrogens involved.[23] Another possible interpretation for the abnormal ΔpK_a is a change in rate-limiting step with changing solvent although this is less likely as a similar shift in pK_a is apparent for acylation, deacylation, methanolysis, and hydrolysis.

The final unusual observation from Table 1 is the inverse KSIE from k_{cat}/K_m for both benzylpenicillin and cephalosporin. This is indicative of a tightening of vibrational frequencies on going to the transition state for acylation which may be due to proton transfer between Tyr-150 and Ser-64 (Chart 1) as the hydrogen-bonded system is modified as covalent bond formation occurs between Ser-64 and the β-lactam carbonyl. This is again consistent with low fractionation factors for the $NH_3^+ - {}^-OAr$ hydrogen bonds compensating for the unexpected small primary isotope effect arising from the breaking of the serine OH bond.

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Table 1. Kinetic Parameters and Solvent Isotope Effects for P99 β-Lactamase C Catalyzed Reactions in H_2O and D_2O at 30 °C and I = 1.0 M (KCl)

	k_{cat}^{max}/K_m (M^{-1} s^{-1})			k_{cat}^{max} (s^{-1})		
substrate (reaction)	H_2O	D_2O	SKIE	H_2O	D_2O	SKIE
benzylpenicillin (hydrolysis)	3.97 (±0.11) × 10^6	4.53 (±0.12) × 10^6	0.88	28.8 (±0.41)	20.3 (±0.61)	1.42
benzylpenicillin (methanolysis)				8.44 (±0.33)[a]	5.94 (±0.43)[a]	1.42
cephaloridine (hydrolysis)	2.48 (±0.09) × 10^6	3.20 (±0.10) × 10^6	0.78	1160 (±45)	782 (±31)	1.48

[a] Units M^{-1} s^{-1}.

Table 2. Apparent pK_a Values from the Kinetic Parameters Indicated for P99 β-Lactamase C Catalyzed Reactions in H_2O and D_2O at 30 °C and I = 1.0 M (KCl)

	k_{cat}/K_m				k_{cat}			
	pK_a^1		pK_a^2		pK_a^1		pK_a^2	
substrate (reaction	H_2O	D_2O	H_2O	D_2O	H_2O	D_2O	H_2O	D_2O
benzylpenicillin (hydrolysis)	5.89 (±0.07)	6.59 (±0.06)	10.13 (±0.06)	10.53 (±0.06)	4.99 (±0.03)	5.83 (±0.07)	9.99 (±0.03)	10.42 (±0.07)
benzylpenicillin (methanolysis)					5.42 (±0.10)	6.14 (±0.18)	9.87 (±0.09)	10.32 (±0.11)
cephaloridine (hydrolysis)	6.29 (±0.04)	7.12 (±0.11)	10.10 (±0.04)	10.42 (±0.04)	5.23 (±0.09)	6.36 (±0.06)	9.24 (±0.11)	9.69 (±0.05)

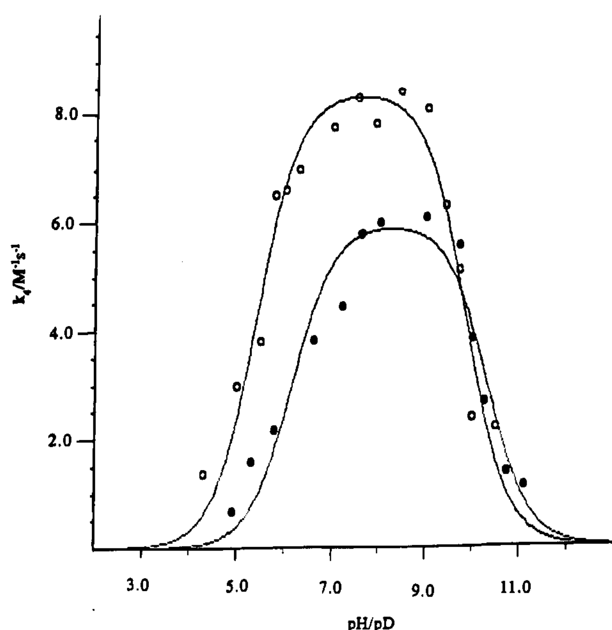
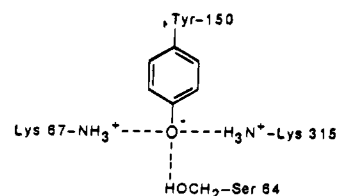


Figure 2. The dependence of the second-order rate constant, k_4, for the methanolysis of benzylpenicillin, catalyzed by *E. cloacae* P99 β-lactamase at 30 °C, on pH (○) and pD (●).

Although there have been several analyses of β-lactamases modified by site-directed mutagenesis their detailed mechanism of action remains controversial. The modification of Lys 67 of the *E. Cloacae* enzyme resulted in lowered acylation rates[24] and Lys 315 His and Lys 315 Gln mutants appear to indicate that the Lys 315 residue is responsible for increasing the nucleophilic properties of another side chain.[25] The activity of the Tyr 150 Phe mutant was decreased for some substrates but retained most of its activity with poor substrates.[15] The interpretation of the kinetic data with mutant enzymes is often quite difficult because the individual microscopic rate and equilibrium constants cannot be determined for good substrates.[1]

Chart 1



By superimposing the active serine side chain and the two oxyanion hole peptide NH groups of the *Citrobacter freundii* class C enzyme on the corresponding groups of chymotrypsin, it has been shown that the Tyr 150 oxygen atom of the β-lactamase is in an equivalent position to the chymotrypsin general base catalytic residue, the imidazole nitrogen of His 57.[2]

Crystallographic data obtained for the *E. Cloacae* class C β-lactamase with a phosphonate monoester inhibitor support this suggestion.[14] The solvent isotope effects reported here are also compatible with Tyr 150 having a severely reduced pK_a because of its relatively positive environment and strong hydrogen bonding of the phenoxide ion by lysine residues. It appears that the Tyr 150 residue is a very strong candidate for the role of a general base catalyst in class C β-lactamases.

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