The Roles of the Carboxy Group in β-Lactam Antibiotics and Lysine 234 in β-Lactamase I

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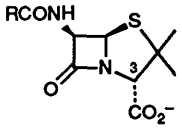
The replacement of the C3 carboxylate in phenoxymethylpenicillin by a hydroxymethyl group and of the C4 carboxylate in cephalosporins by both a lactone and an aldehyde gives derivatives which are still good substrates for *Bacillus cereus* 569/H β-lactamase I. The enzyme rate-enhancement factors for the hydrolysis of the modified β-lactams vary from 10^4 to 10^6. All three modified substrates show bell-shaped (k_{cat}/K_m)–pH profiles indicative of two catalytically important ionising residues on the protein of pK_a about 5 and 9. Although lysine 234 is a highly conserved residue in class A β-lactamases and has been traditionally thought to interact with the carboxylate of the β-lactam antibiotic, it is not responsible for the decrease in enzyme activity at high pH corresponding to the pK_a of about 9.

Most β-lactam antibiotics contain a negatively charged substituent which is thought to be essential for molecular recognition. There is a carboxylate group at C3 in penicillins (**1**) and at C4 in cephalosporins (**2**).[1]



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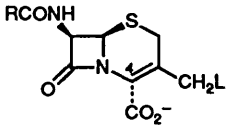

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The β-lactam antibiotics interact with at least two classes of enzyme, one of which is the killing target for their antibacterial activity, the transpeptidases, and the other is the major cause of antibacterial resistance, the β-lactamases.[2] Four major classes of β-lactamase have been identified of which the class A enzymes have been characterised in most detail. In class A β-lactamases the lysine 234 residue is conserved in 19 out of the 20 known sequences. The exception has a positively charged arginine residue at this point.[3] The amine side chain of Lys-234 points into the presumed active site and, in its positively charged form, is thought to form an electrostatic interaction with the carboxylate group in penicillins (**1**) and cephalosporins (**2**),[4] (Fig. 1). The rate of the enzyme-catalysed hydrolysis of β-lactams normally displays a bell-shaped pH-dependence.[5] It has generally been assumed that the decreased activity at high pH is due to the deprotonation of lysine 234 which thus reduces the electrostatic interaction with the anionic carboxylate.[6]

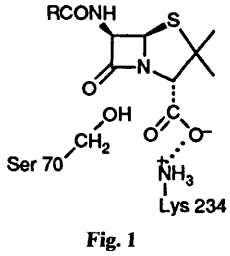
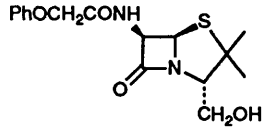


Fig. 1

There are two simple ways to test this belief—one is to change the carboxylate substituent in the β-lactam antibiotic and the other is to change the lysine substituent in the protein. The latter method has recently been reported for two β-lactamases.[7,8]



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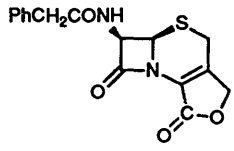

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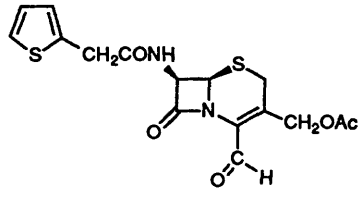

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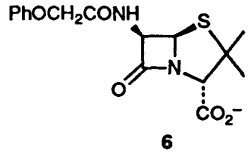
Herein, we report the effect on *Bacillus cereus* 569/H β-lactamase I activity of replacing the carboxylate by a hydroxymethyl group in penicillins (**3**) and by a lactone (**4**) and an aldehyde (**5**) in cephalosporins. In addition to removing the negative charge the aldehyde residue provides the possibility of covalent bond formation through imine formation with a suitably placed amine residue in β-lactamase. A preliminary account of the reactivity of the lactone **4** has been reported.[9]



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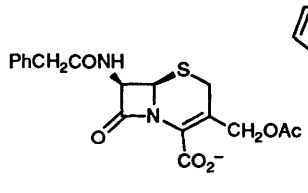

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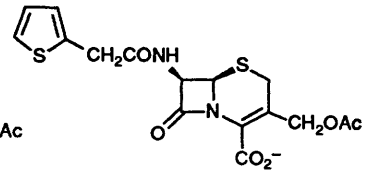

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Experimental

Kinetics.—As previously described.[10]

Synthesis. (3-*Hydroxymethyl*)*phenoxymethylpenicillin* **3**.[11]—Phenoxymethylpenicillin (5.0 g, 14.3 mmol) was stirred in anhydrous tetrahydrofuran (THF) (40 cm^3) and cooled to −10 °C. To this was added triethylamine (1.45 g, 14.3 mmol) dropwise with stirring, followed by a solution of ethyl chloroformate (1.55 g, 14.3 mmol) in 15 cm^3 of anhydrous THF. The reaction was stirred for 2 h at −10 °C, after which sodium borohydride (1.08 g, 28.6 mmol) was added in small quantities over 10 min. The reaction mixture was then brought slowly to room temperature and left for 30 min. Water (100 cm^3) was then added and the product alcohol extracted with dichloromethane (3 × 50 cm^3), and the combined extracts were washed with iced saline solution, filtered and then dried over anhydrous magnesium sulfate. Reduction of the volume under vacuum and treatment with acetone–hexane produced a crystalline product. The isolated product was purified by dissolution in a small quantity of dichloromethane and chromatographed through a silica gel column, eluting first with dichloromethane and then ethyl acetate. ν_{max}(Nujol)/cm^{-1} 1779.0, 1686.6 and 3370.5; δ(DMSO): 1.50 (s, 6 H, 2 × $-CH_3$), 3.5 (m, 2 H, 3 × $-CH_2$), 4.6 (s, 2 H, PhOCH_2), 4.9 (t, 1 H, OH, J = 5.4 Hz), 5.3 (d, 1 H, H-5), 5.4 (dd, 1 H, H-6), 3.75 (t, 1 H, H-3, J = 7.2 Hz), 6.9 (m, 3 H, ArH), 7.3 (t, 2 H, aromatic H) and 8.55 (d, 1 H, NH, J = 7.7 Hz). Analysis: (Found: C, 57.2; H, 5.9; N, 8.4. Calc. for $C_{16}H_{20}N_2O_4S$: C, 57.14; H, 5.95; N, 8.33%); m.p. 129–131 °C (lit.,[11] 128–130 °C).

3-*Acetoxymethyl*-4-*hydroxymethyl*-7β-(2-*thienylacetamido*)-3-*cephem*.[12]—To a stirred suspension of the sodium salt of 7β-(2-thienylacetamido)cephalosporanic acid (10 g, 24 mmol) in a mixture of anhydrous dichloromethane (140 cm^3) and dimethylformamide (0.5 cm^3) at 0 °C, was slowly added a solution of oxalyl chloride (7.95 g, 62 mmol) in anhydrous dichloromethane (12 cm^3). As the reaction proceeded, the starting material gradually went into solution. After 1 h, the solvent was evaporated off at a temperature below 15 °C. The 7β-(2-thienylacetamido)cephalosporanyl chloride was not isolated but taken up in anhydrous tetrahydrofuran (110 cm^3) and the temperature taken down to 0 °C. Lithium tri-*tert*-butoxyaluminium hydride (8.77 g, 345 mmol) in anhydrous tetrahydrofuran (70 cm^3) was then added to the solution over a period of 20 min. After being stirred for a further 30 min, the mixture was poured into a chilled solution of dilute hydrochloric acid (pH 1) and the solution was adjusted to pH 2. The reaction mixture was then extracted with ethyl acetate and the extract was washed with a 5% aqueous sodium hydrogen carbonate followed by saturated brine. The solvent was then evaporated off to yield the crude product. The mixture was then chromatographically purified using a silica column and an eluent system of 7:3 ethyl acetate:diethyl ether. Evaporation of the solvent gave the alcohol (1.25 g, R_f 0.4, m.p. 172–173 °C).

$\delta(CDCl_3)$: 2.08 (3 H, s, CH_3CO) 3.25, 3.53 (2 H, AB, J = 17.5 Hz, H-2), 3.53 (1 H, m, CH_2OH), 3.88 (2 H, s, CH_2CONH), 4.36, 4.56 (2 H, AB, J = 13.5 Hz, CH_2OC), 4.99 (1 H, d, J = 4.8 Hz, H-6), 5.79 (1 H, dd, J = 4.9, 9.3 Hz, H-7), 6.33 (1 H, d, J = 9.3 Hz) and 7.01–7.29 (3 H, m, thiophene H); ν_{max}(Nujol)/cm^{-1} 3360, 1730, 1656, 1530 and 1371.

3-*Acetoxymethyl*-7β-(2-*thienylacetamido*)-3-*cephem*-4-*carbaldehyde* (**5**).[13]—3-Acetoxymethyl-4-hydroxymethyl-7β-(2-thienylacetamido)-3-cephem (500 mg, 1.325 mmol) and dicyclohexylcarbodimide (DCC) (900 mg, 4.375 mmol) were added to freshly distilled dimethyl sulfoxide (DMSO) (12.5 cm^3). To this solution was added freshly distilled dichloroacetic acid (100 μl) with stirring. After a period of 10 min the solution was observed to pass from a pale orange/brown colour to dark green and then finally to dark brown. Precipitation of a white solid (dicyclohexylurea) was also apparent.

The aldehyde was separated from the brown solution by preparative reversed-phase HPLC using an eluent system of 30% acetonitrile–70% distilled water, the detectors being set to monitor at a wavelength of 254 nm and an elution rate of 12 cm^3 min^{-1}. The acetonitrile in the eluent mixture containing the aldehyde was removed by evaporation and the remaining water was removed by freeze-drying. Evaporation of the water yielded the aldehyde as a white solid (m.p. 132–134 °C). $\delta(CDCl_3)$: 2.10 (3 H, s, OAc), 3.45, 3.60 (2 H, AB, J = 18.5 Hz, H-2), 3.87 (2 H, s, CH_2CONH), 4.97 (1 H, d, J = 5.0 Hz, H-6), 5.05 (2 H, s, CH_2OAc), 5.88 (1 H, dd, J = 5.0, 8.6 Hz, H-7), 6.55 (1 H, d, J = 8.6 Hz, NH), 6.99–7.27 (3 H, m, thiophene-H) and 9.94 (1 H, s, CHO); ν_{max}(Nujol)/cm^{-1} 1775, 1745, 1700 and 1665.

Results and Discussion

Investigating the efficiency of enzyme catalysis invariably involves determining the effect of modifying the substrate, the enzyme or the reaction conditions on reactivity. Such modifications can affect the free energies of the ground state and the transition state and changes in rate constants only reflect the *difference* in energies between these two states. Furthermore, changes in reactivity can be the result of differences in intrinsic 'chemical' effects or in binding interactions between the substrate and enzyme.[14] The effects of changes in substrate structure on enzyme activity are often used to identify specific binding sites between parts of the enzyme and substrate. These changes can affect the ease of bond-making and -breaking by 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 important to separate these two effects before conclusions about specific binding sites can be made. We recently suggested[9] that an 'enzyme rate-enhancement factor' (EREF) could be evaluated by dividing the second-order rate constant for the enzyme-catalysed reaction, k_{cat}/K_m, by that for the hydrolysis of the same substrate catalysed by hydroxide ion, k_{OH}. Replacing the carboxylate group in penicillins (**1**) and cephalosporins (**2**) by a neutral substituent increases the rate of alkaline hydrolysis. The second-order rate constant k_{OH} is twice as large for **3** compared with benzylpenicillin and 130 times and 26 times greater for **4** and **5**, respectively, compared with the analogous cephalosporin with a carboxy group at C4 (Table 1). This is expected from previous observations which have shown that electron-withdrawing substituents attached to the β-lactam carbonyl carbon towards nucleophilic attack and increase the ease of C–N bond cleavage.[15]

(i) *Hydrolysis of the Penicillin* C3-*Alcohol* (**3**).—Two aspects of the β-lactamase I catalysed hydrolysis of the penicillin C3-alcohol **3** are important. Firstly, the absolute reactivity as measured by k_{cat}/K_m, which at pH 7 is 4.59 × 10^5 dm^3 mol^{-1} s^{-1}. This is 100 times less than that shown by phenoxymethylpenicillin **6** at the same pH. However, the penicillin C3-alcohol is still a good substrate—much better than most cephalosporins, for example. Nonetheless, the enzyme rate-enhancement factor (EREF), using the second-order rate constant for alkaline hydrolysis as a measure for chemical reactivity, is much less for the C3-alcohol, 1.5 × 10^6, than that for the C3-carboxylate, 2.6 × 10^8. The difference in EREF is slightly greater than the hundredfold ratio of the values of k_{cat}/K_m because the β-lactam of the C3-alcohol (**3**) is twice as susceptible to nucleophilic attack by hydroxide ion as that of the C3-carboxylate (**6**). The hydroxymethyl group is more electron withdrawing than the carboxylate group which increases both the electrophilicity of the β-lactam carbonyl carbon and the leaving-group ability of the β-lactam amino group.

Table 1 Kinetic data for the hydrolysis of β-lactam derivatives catalysed by β-lactamase I and hydroxide ion at 30.0 °C in 1% v/v dioxane unless otherwise stated

	$(k_{cat}/K_m)_{max}$/dm^3 mol^{-1} s^{-1}	k_{OH}/dm^3 mol^{-1} s^{-1}	EREF	pK_1	pK_2
Phenoxymethylpenicillin C3-alcohol **3**	4.59×10^5	0.310	1.5×10^6	5.1 ± 0.2	9.4 ± 0.2
Phenoxymethylpenicillin **6**	4.45×10^7	0.174	2.6×10^8	4.9 ± 0.1	8.8 ± 0.1
Cephalosporin lactone **4**	3.23×10^5	11.6	2.8×10^4	4.8 ± 0.2	9.2 ± 0.2
Benzylcephalosporin **7**	5.96×10^3	8.90×10^{-2}	6.7×10^4	4.4 ± 0.1	8.8 ± 0.1
Cephalothin C4-aldehyde **5**[a]	1.06×10^6	2.25	4.7×10^5	6.5 ± 0.1	9.8 ± 0.1
Cephalothin **8**	7.46×10^3	8.5×10^{-2}	8.8×10^4	4.7 ± 0.1	9.1 ± 0.1

[a] In 10% v/v butane-1,4-diol–water.

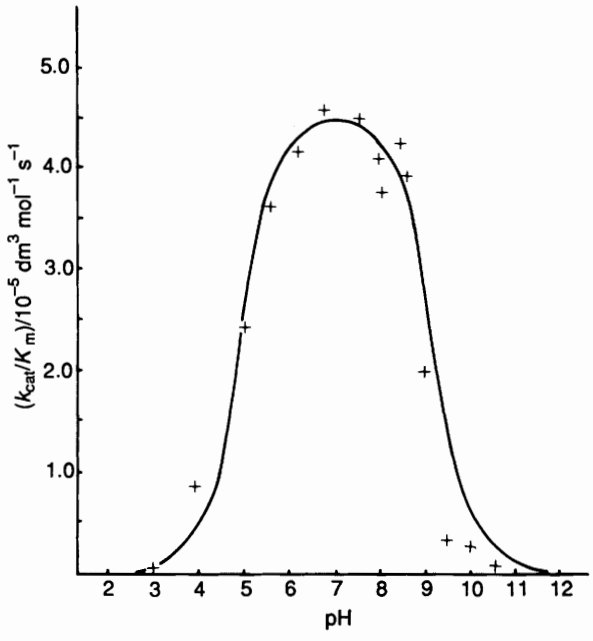

Fig. 2 A plot of the observed second-order rate constant (k_{cat}/K_m)/dm^3 mol^{-1} s^{-1} against pH for the β-lactamase I catalysed hydrolysis of the C3-hydroxymethyl phenoxymethylpenicillin **3** in water at 30 °C

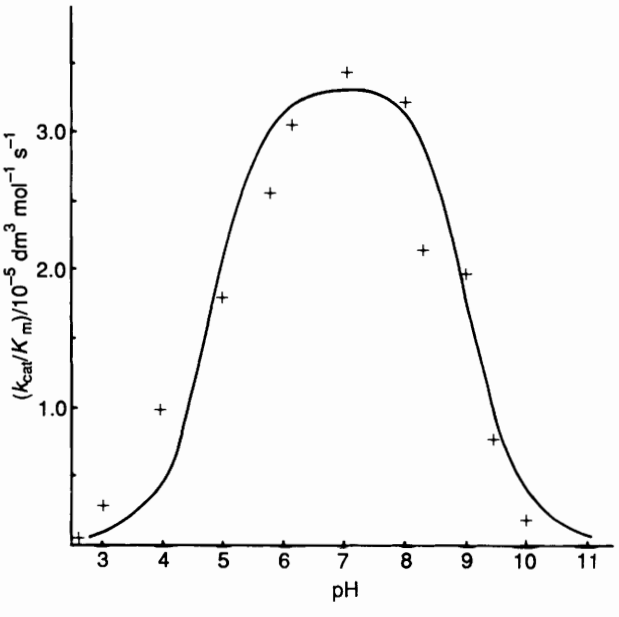

Fig. 3 A plot of the observed second-order rate constant (k_{cat}/K_m)/dm^3 mol^{-1} s^{-1} against pH for the β-lactamase I catalysed hydrolysis of the cephalosporin lactone **4** in water at 30 °C

The second important aspect is the pH–rate profile for the enzyme-catalysed hydrolysis of the penicillin C3-alcohol **3** which is shown in Fig. 2. The values of k_{cat}/K_m show the normal bell-shaped dependence upon pH indicative of two important ionisations, K_1 and K_2 in eqn. (1), corresponding to pK_a values

$$\frac{k_{cat}}{K_m} = (k_{cat}/K_m)_{max} \cdot \frac{K_1(H^+)}{K_1K_2 + K_1(H^+) + (H^+)^2} \quad (1)$$

$$= \frac{(k_{cat}/K_m)_{max}}{\frac{(H^+)}{K_1} + 1 + \frac{K_2}{(H^+)}}$$

of 5.10 and 9.40, respectively. The high-pK_a group has to be in its acidic protonated form for maximum enzyme activity, $(H^+) > K_2$. The low-pK_a group has to be in its basic non-protonated form for maximum enzyme activity, $(H^+) < K_1$. The pH dependence of k_{cat}/K_m indicates the ionisations in the free enzyme and substrate which are important for catalysis. It is often assumed that the high pK_a group observed in the β-lactamase A catalysed hydrolysis of β-lactam antibiotics is due to Lys-234.[5–7] The role of this lysine residue, in its positively charged form, is considered to be the electrostatic interaction with the negatively charged carboxylate on the antibiotic (Fig. 1). It seems unlikely that replacement of the carboxylate group by a neutral hydroxymethyl would result in an interaction with a similar pH dependence. The simplest conclusion therefore is that the ionisable group of pK_a = 9.40 is serving a function other than interacting with the C3-carboxylate of penicillins.

(ii) *Hydrolysis of the Cephalosporin Lactone* (**4**).—We have previously reported that the lactone **4** is a substrate for β-lactamase I and that the enzyme-catalysed hydrolysis opens the β-lactam ring to give a product with the lactone still intact.[9] The data are given in Table 1 which show that the enzyme rate-enhancement factor is 3×10^4. The cephalosporin lactone **4** is a 50-times better substrate than the corresponding cephalosporin **7** with a free carboxylate residue.

The plot of k_{cat}/K_m against pH (Fig. 3) also generates the normal bell-shaped dependence indicative of two important ionisations, eqn. (1). In this case, the two corresponding pK_a values are 4.8 and 9.2 (Table 1). This observation could suggest that the lactone is a suitable mimic for the carboxylate anion and that it also interacts with a positively charged residue on the protein and that this favourable interaction decreases with increasing pH.[9] A simpler interpretation, and one that agrees more readily with the other observations reported herein, is that the descending limb of the pH–rate profile at high pH is not due to the deprotonation of a residue which is responsible for binding the carboxylate group on normal β-lactam antibiotics.

(iii) *Hydrolysis of the Cephalothin* C4-*Aldehyde* (**5**).—Replacement of the C4-carboxylate of cephalothin by the neutral aldehyde group in **5** also gives a β-lactam which is a good substrate for β-lactamase I. The second-order rate constant k_{cat}/K_m for the enzyme-catalysed hydrolysis at pH 8 is 1.0×10^6

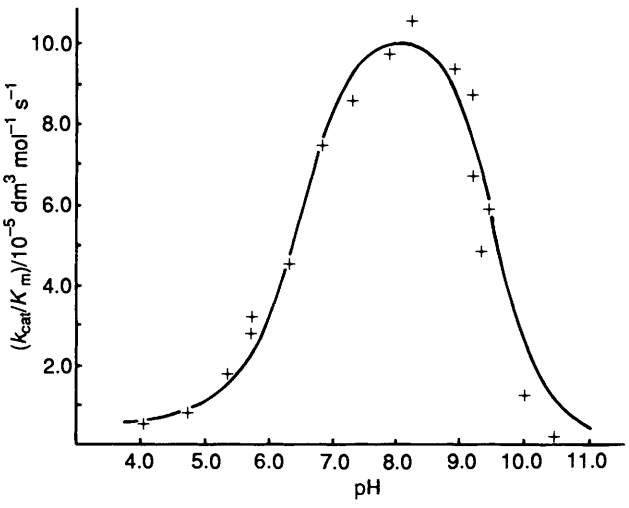

Fig. 4 A plot of the observed second-order rate constant (k_{cat}/K_m)/dm^3 mol^{-1} s^{-1} against pH for the β-lactamase I catalysed hydrolysis of the C4-cephalothin aldehyde **5** in water at 30 °C

dm^3 mol^{-1} s^{-1}. This is, in fact, 134 times larger than that for the corresponding natural cephalothin **8** with a C4 carboxylate! Similar to the situation with the cephalosporin lactone **4**, replacement of the carboxylate group can lead to an increase in enzyme activity. The enzyme rate-enhancement factor for the C4-aldehyde **5** is 4.7×10^5 compared with a value of 8.8×10^4 for cephalothin itself. The pH–rate profile for the β-lactamase catalysed hydrolysis of the cephalosporin aldehyde **5** shows the normal bell-shaped behaviour (Fig. 4). However, there may be some indications that at low pH the rate does not fall off as

$$\begin{array}{ccccc} EH_2 & \overset{K_1}{\rightleftharpoons} & EH & \overset{K_2}{\rightleftharpoons} & EH \\ \downarrow k_{H_2} & & \downarrow k_{H_2} & & \\ \text{prods} & & \text{prods} & & \end{array}$$

Scheme 1

rapidly as predicted by eqn. (1). Empirically, the data gives a better fit to Scheme 1 and the corresponding eqn. (2). It makes

$$\frac{k_{cat}}{K_m} = \frac{k_{H_2}(H^+)^2 + k_{H_1}K_1(H^+)}{K_1K_2 + K_1(H^+) + (H^+)^2} \quad (2)$$

little difference to the pK_a values which scheme is used. The two pK_a values determined from these results are 6.5 and 9.8.

These observations also indicate that the high pK_a group on the protein which is affecting reactivity is not one which is interacting with the anionic carboxylate group on penicillins and cephalosporins. We are forced to the conclusion that although lysine 234 is a highly conserved residue of the serine β-lactamases it is not the functional group which causes the decrease in activity at high pH.

The decrease in activity at high pH could be due to the ionisation of another group—the most likely candidates are lysine-73 or tyrosine-105. The latter is located near the active site although its nitration does not result in the loss of enzyme activity at pH 7.[16] It seems probable that the importance of this group would be in contributing to the enzyme's conformation rather than a direct catalytic function. It has been suggested that protonated lysine-73 stabilises the anionic tetrahedral intermediate[17] but the pK_a of this group is thought to be >9.[18]

It seems improbable that the ionisation at high pH is due to a catalytic group acting as a proton donor to the β-lactam nitrogen in the breakdown of the tetrahedral intermediate as the pK_a of the thiazolidine nitrogen in the hydrolysis product is 5.2 and it is anticipated that a strongly acidic proton-donating group is therefore required for ring opening.[1,2]

By site-directed mutagenesis, the lysine-234 residue of the *Bacillus licheniformis* class A β-lactamase has been replaced by alanine and glutamic acid which resulted in a decrease in k_{cat}/K_m of a factor of 9000 and 3600, respectively, for benzylpenicillin as the substrate.[7] Interestingly, the pH–rate profiles for the mutated enzymes still display a bell-shape, the pK_2 values being 8.6, 8.6 and 10.0 for the wild, glutamic 234 and alanine 234 mutants, respectively. It is thus clear that pK_2 *is not due to the ionisation of lysine 234.*

Lysine-234 has also been replaced by histidine in the β-lactamase A from *Streptomyces albus* G.[8] The wild-type shows the normal bell-shaped k_{cat}/K_m–pH profile for the hydrolysis of benzylpenicillin with pK_1 and pK_2 values of about 5.0 and 9.9, respectively. The histidine-234 mutant enzyme shows the same maximum value for k_{cat}/K_m but is shifted to a lower pH as pK_2 is now 6.3. It is suggested that this new pK_2 value reflects the ionisation of the newly introduced histidine 234. However, another interpretation is that pK_2 is not due to the ionisation of the residue at 234 but is perturbed by the nature of the residue at that position. Yet another possibility is that pK_2 is a kinetic pK_a. Interestingly, we have found that the value of pK_2, determined from the pH dependence of k_{cat}/K_m varies with the nature of the substrate.[19] It therefore seems unlikely that pK_2 represents the ionisation of one particular residue in the enzyme.

Replacement of Lys-234 by arginine in the Gram-negative β-lactamase TEM-1 gives an enzyme with similar activity toward cephalosporins and most penicillins.[20] Substituting the Lys-234 by threonine gives an enzyme with only a tenfold reduction in activity towards penicillins at high pH although activity towards cephalosporins is almost completely removed.[20]

An analysis of the hydration patterns in the *B. licheniformis* enzyme shows that the side-chain amino group of Lys-234 is bound to a strongly conserved water molecule which would be difficult to displace by the substrate carboxylate.[21] An alternative orientation of the substrate in the active site cavity could involve the β-lactam carboxylate interacting with a positively charged arginine.[22,23] This would be in common with other systems adopting this strong interaction.[24]

An unsolved problem associated with β-lactams, in general, is that they do not undergo ring opening as readily as would be suggested by the strain energy of the four-membered ring.[1,25] One possible explanation is that unlike acyclic systems in which carbon–nitrogen bonds are broken by a stretching motion, four-membered rings may be opened by rotation.[25,26] A consequence of this would be that substituents attached to the incipient amino group would move considerably upon ring opening the β-lactam. It would then be inappropriate to have the substrate's carboxylate tightly bound to the enzyme.

Finally, it is worth noting that the cephalosporin C4 aldehyde **5** does not act as an inhibitor of β-lactamase. It is conceivable that if the 'natural' carboxylate on the β-lactam antibiotic interacts with a lysine residue then its replacement by an aldehyde could lead to imine Schiff base formation.[27] However, the aldehyde **5** is an α,β-unsaturated ketone and it is possible that even if the aldehyde of the antibiotic and an amine residue of the proteins are held in close proximity in the enzyme–substrate complex the aldehyde carbonyl group is insufficiently active to form an imine. The alternative interpretation, of course, is that the two residues are not held in close proximity or that hydrolytic turnover is faster than imine formation.

Acknowledgements

We thank the SERC (N. L.) and the NAB (D. P.) for support.

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Paper 2/04642C
Received 28*th August* 1992
Accepted 12*th October* 1992