The pH-dependence of class B and class C β -lactamases

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The classification by structure allots β -lactamases to (at present) three classes, A, B and C. The pH-dependence of the kinetic parameters for class B and class C have been determined. They differ from each other and from class A β -lactamases. The class B enzyme was β -lactamase II from *Bacillus cereus* 569/H/9. The plots of k_{cat} against pH for the hydrolysis of benzylpenicillin by Zn(II)-requiring β -lactamase II and Co(II)-requiring β -lactamase II were not symmetrical, but those of k_{cat}/K_m were. A similar feature was observed for the hydrolysis of both benzylpenicillin and cephalosporin C by a class C β -lactamase from *Pseudomonas aeruginosa*. The results have been interpreted by a scheme in which two ionic forms of an intermediate can give product, but do so at differing rates.

β -Lactamases are widely distributed bacterial enzymes that catalyse the hydrolysis of the β -lactam ring present in penicillins, cephalosporins and related antibiotics. These enzymes can play an important part in the resistance of pathogens to antibiotics, and they are numerous and diverse. Work on their structure has reached the stage of recognizing three distinct classes, A, B and C (Ambler, 1980; Jaurin & Grundström, 1981). The Zn(II)-requiring enzyme from *Bacillus cereus* (Sabath & Abraham, 1966; Davies & Abraham, 1974), β -lactamase II, is to date the sole representative of class B. Only one other β -lactamase is known to require a metal ion for activity; this is the Zn(II)-requiring β -lactamase from *Pseudomonas maltophilia* (Saino *et al.*, 1982), a pathogen that is resistant to many β -lactam antibiotics. The chromosomally encoded β -lactamases from *Escherichia coli* (Lindström *et al.*, 1970; Jaurin & Grundström, 1981) and from *Pseudomonas aeruginosa* (Sabath *et al.*, 1965; Berks *et al.*, 1982; Knott-Hunziker *et al.*, 1982a) (referred to hereafter as the *Pseudomonas* β -lactamase) belong to class C. The distinction between β -lactamases belonging to classes A, B and C is functional as well as structural, and there exist important differences in their specificities and their susceptibilities to inhibitors. Little is known of the kinetics of class B and class C β -lactamases, and in particular the pH-dependence of their kinetic parameters has not been studied, although this dependence is known for a class A β -lactamase (Waley, 1975). The work reported in the present paper fills this gap. The forms of some of the curves (see Fig. 1 or Fig. 4) suggest that there are two ionic forms of intermediates that

give products at different rates. Both Zn(II)- and Co(II)-requiring β -lactamase II have been studied and there are significant differences in the pK values that characterize the kinetic parameters.

Materials and methods

Materials

β -Lactamase II from *Bacillus cereus* 569/H/9 was prepared as described previously (Davies *et al.*, 1974; Baldwin *et al.*, 1980b), as was the β -lactamase from a mutant of *Pseudomonas aeruginosa* 18S (Flett *et al.*, 1976; Knott-Hunziker *et al.*, 1980; Berks *et al.*, 1982). The apoenzyme of β -lactamase II was prepared by dialysis against 10 mM-EDTA/0.1 M-sodium succinate, pH 6, for 18 h at 4°C and then adsorption on Sephadex CM50 in a Pasteur pipette. The Sephadex had been previously equilibrated with 0.1 M-sodium succinate, pH 6. The column was washed with 5 vol. of 0.1 M-sodium succinate (pH 6) to remove EDTA. The apoenzyme was eluted with 1 M-NaCl in 0.1 M-sodium succinate; it had lost 99.9% of activity (when measured with benzylpenicillin as substrate, at pH 6). Washing all buffers and glassware with dithizone in chloroform did not alter this value and was subsequently omitted in the preparation of apoenzyme. Stock solutions of Co(II)-requiring β -lactamase II were prepared as required by making a solution of apoenzyme 1 mM in CoCl_2 ; Co(II)-requiring β -lactamase II, unlike the Zn(II) enzyme or the apoenzyme, loses activity at 4°C over a period of days. The Co(II)-requiring β -lactamase II had 13% of the activity of the Zn(II)-requiring enzyme

(Baldwin *et al.*, 1980b), and so the 0.1% remaining in the apoenzyme did not contribute significantly to the activity of the Co(II)-requiring enzyme.

Methods

The initial rate of hydrolysis of benzylpenicillin was measured in the pH-stat in 0.5 M-NaCl at 30°C; for the direct determinations of k_{cat} (the limiting rate at very high concentrations of substrate and unit concentration of enzyme) the concentration of substrate was about 30 times the Michaelis constant for Zn(II)-requiring β -lactamase II and about 750 times for Co(II)-requiring β -lactamase II. The concentrations of the enzymes were about 20 nM and about 200 nM respectively. The concentrations of Zn(II) and Co(II) ranged from 10 μ M to 100 μ M, and from 100 μ M to 1 mM respectively, lower concentrations being used at higher pH values. The rates were not limited by the concentrations of Zn(II) or Co(II). Assays were carried out in triplicate; the mean value of the initial rate was corrected to allow for two factors. First, non-enzymic hydrolysis was significant at pH values greater than 6, and was far more marked when Zn(II), as opposed to Co(II), was present (cf. Gensmantel *et al.*, 1978). Secondly, correction was made for buffering by the product, penicilloic acid, at pH values less than 6, as described by Kurtz & Niemann (1962). The initial rate of hydrolysis of cephalosporin C by the *Pseudomonas* β -lactamase was measured in the pH-stat in 0.1 M-NaCl at 30°C, at concentrations of approx. 100 times the Michaelis constant, for the direct determination of k_{cat} .

Progress curves for the hydrolysis of benzylpenicillin by β -lactamase II were measured spectrophotometrically at 232 nm (Waley, 1974). A Cary model 219 spectrophotometer was used, with a pathlength of 1–10 mm. The following buffers were used, all in 0.5 M-NaCl; 0.2 M-succinic acid/sodium succinate (pH 5–6); 0.2 M-Mops (4-morpholinepropanesulphonic acid; sodium salt) (pH 6.5–8); 0.1 M-boric acid/sodium borate (pH 8.5–10). The concentrations of Zn(II) and Co(II) were similar to those specified above for the pH-stat experiments. No loss of enzymic activity from either Zn(II) or Co(II) enzyme was detected at the extreme pH values by Selwyn's (1965) method. The progress curves were analysed by the half-time method of Wharton & Szawelski (1982), and, after a check for linearity, with the program of Cornish-Bowden for a direct linear plot based on the method of Cornish-Bowden & Eisenthal (1978).

Progress curves for the hydrolysis of cephalosporin C by the *Pseudomonas* β -lactamase were measured at 259.7 nm. The concentrations of substrate and enzyme were 50–400 μ M and about 2 nM respectively. The following buffers were used, all in 0.1 M-NaCl: 20 mM-Mes (4-morpholine-ethanesul-

phonic acid) (pH 5–6); 20 mM-Mops (pH 7–8); 20 mM-NaHCO₃/Na₂CO₃ (pH 9–10). Initial rates, measured by the 'chord' method (Waley, 1981), were used to determine k_{cat} and K_m by the program of Cornish-Bowden referred to above. The hydrolysis of benzylpenicillin (200 μ M) by the *Pseudomonas* β -lactamase was measured spectrophotometrically in a cuvette with pathlength 1 mm to obtain k_{cat} . The K_m was so low that it was more convenient to use the method of competing substrates (Waley, 1983). The effect of benzylpenicillin (30 μ M or 60 μ M) on the hydrolysis of cephalosporin C (180 μ M) by the *Pseudomonas* β -lactamase (about 3 nM) was measured (at 259.7 nm); the progress curves were compared with those from the hydrolysis of cephalosporin C alone. Values of k_{cat}/K_m of benzylpenicillin, relative to k_{cat}/K_m of cephalosporin C, were obtained; the value of k_{cat} was also obtained, and agreed with that obtained directly.

Fitting equations to data

The bell-shaped curves reflecting the pH-dependence of k_{cat}/K_m were fitted to eqn. (1):

$$k_{\text{obs}} = (\tilde{k}_{\text{cat}}/\tilde{K}_m)/(1 + 10^{pK_1 - \text{pH}} + 10^{\text{pH} - pK_2}) \quad (1)$$

A least-squares fit was used, with a linearization (Taylor's series) procedure based on that of Wiberg (1974), adjustments being made to pK_1 and pK_2 . For any pair of values of pK_1 and pK_2 , the value of $\tilde{k}_{\text{cat}}/\tilde{K}_m$ was found by least-squares. The pH-dependence of k_{cat} gave curves with 'tails' and these were fitted to eqn. (2) (see also Scheme 1):

$$k_{\text{cat}} = [k_{+2} + k'_{+2}(10^{\text{pH} - pK'_1})]/f \quad (2)$$

where

$$f = 1 + 10^{pK'_1 - \text{pH}} + (10^{\text{pH} - pK'_2})(1 + 10^{\text{pH} - pK'_1})$$

with a program based on 'GRIDLS' of Bevington (1969) (see also Holt & Norris, 1977). The method utilized a direct-search algorithm to find the best (in the least-squares sense) pK values; k_{cat} is a non-linear function of the three pK values, but a linear function of k_{+2} and k'_{+2} . For a choice of the pK values, the values of k_{+2} and k'_{+2} were directly determined in the program by linear regression. The same final values were obtained from different starting values of the pK values. Both programs were implemented on a PDP-11 computer.

Results and discussion

β -Lactamase II

The plot of k_{cat} against pH for the hydrolysis of benzylpenicillin by Zn(II)-requiring β -lactamase differs from the usual bell-shaped curve by showing

a 'tail' on the alkaline side (Fig. 1). The evidence that this is not an artefact is as follows. The non-enzymic hydrolysis of benzylpenicillin has been allowed for, and moreover the curve has the same shape for the reaction at 0°C, where non-enzymic hydrolysis is negligible. All the experiments were carried out at saturating concentrations of metal ion. The corresponding plot for the Co(II)-requiring β -lactamase II (Fig. 2) had the same shape.

The pH-dependence of the observed k_{cat} suggests that there are two ionic forms of the reactive complex that can react further to form product. The simplest scheme thus demands four ionic forms linked by three ionizations (Scheme 1). The ob-

served k_{cat} is then given by eqn. (3) (Laidler & Bunting, 1973; Dworschack & Plapp, 1977):

$$k_{\text{cat}} = \frac{k_{+2} + k'_{+2} \cdot \frac{K'_2}{[\text{H}^+]}}{\frac{[\text{H}^+]}{K'_1} + 1 + \frac{K'_2}{[\text{H}^+]} + \frac{K'_2 K'_3}{[\text{H}^+]^2}} \quad (3)$$

which gave a good fit to the experimental plots (Figs. 1 and 2). On the other hand, the pH-dependence of k_{cat}/K_m can be accounted for by three ionic forms (linked by two ionizations) (Fig. 3). When both proton-transfer reactions and substrate-binding are fast relative to turnover, the observed k_{cat}/K_m is given by eqn. (4) (Laidler & Bunting,

Fig. 1. The pH-dependence of k_{cat} for the hydrolysis of benzylpenicillin by Zn(II)-requiring β -lactamase II. The rate of hydrolysis was measured in the pH-stat in 0.5 M-NaCl at 30°C (●) and at 0°C (○). The curves are theoretical (see eqns. 1 and 2).

Fig. 2. The pH-dependence of k_{cat} for the hydrolysis of benzylpenicillin by Co(II)-requiring β -lactamase II. The rate of hydrolysis was measured in the pH-stat (●) or spectrophotometrically (○) in 0.5 M-NaCl at 30°C. The curve is theoretical.

Scheme 1. Model for the pH-dependence of class B and class C β -lactamases

Fig. 3. The pH-dependence of k_{cat}/K_m for the hydrolysis of benzylpenicillin by Co(II)-requiring β -lactamase II. The rate of hydrolysis was measured in the pH-stat (●) or spectrophotometrically (○) in 0.5 M-NaCl at 30°C. The curve is theoretical (see eqns. 1 and 2).

1973; Brocklehurst & Dixon, 1976; Dixon & Webb, 1979):

$$\frac{k_{\text{cat}}}{K_m} = \frac{\tilde{k}_{\text{cat}}}{\tilde{K}_m} \left(1 + \frac{[\text{H}^+]}{K_1} + \frac{K_2}{[\text{H}^+]} \right) \quad (4)$$

The values in Table 1 are consistent with dissociation of substrate being faster than formation of product. Thus, if $K_s \geq 10^{-3} \text{ M}$ and $k_{\text{on}} \geq 10^{-9} \text{ M}^{-1} \cdot \text{min}^{-1}$ then $k_{\text{off}} \geq 10^6 \text{ min}^{-1}$, whereas $k_{+2} = 5 \times 10^4 \text{ min}^{-1}$; K_s , k_{on} and k_{off} are the dissociation constant of enzyme-substrate complex, and the rate constants for formation and dissociation of the complex respectively. The limits cited are based on the observed values of K_m and k_{cat}/K_m . The kinetic parameter k_{cat}/K_m characterizes the formation of complex from enzyme and substrate, and k_{cat} characterizes the decomposition of complex to product, and their differing pH-dependence is not uncommon.

The pK values obtained are given in Table 1. The pK values from the initial rate of hydrolysis of benzylpenicillin by Zn(II)-requiring β -lactamase II measured in the pH-stat agreed with those from progress curves measured spectrophotometrically to about 0.2 of a pH unit (Table 1).

A group detected by n.m.r. studies of the Zn(II) ligands (Baldwin *et al.*, 1978) had a pK (5.2) fairly close to the value (5.5) that characterizes the pH-dependence of k_{cat}/K_m for Zn(II)-requiring β -lactamase II. This is consistent with the view that the Zn(II) of the mono-zinc enzyme forms part of the

active site. The difference between the values of 5.2 and 5.5–5.6 is not regarded as significant, as the solvents ($^2\text{H}_2\text{O}$ and H_2O) and temperature differed.

The role of the Zn(II) in β -lactamase II may be to interact with the oxygen atom of the β -lactam or to provide, as a nucleophilic group, metal-bound water. The possibility of reverse protonation, suggested for carboxypeptidase A (Cleland, 1977), can be excluded if the two pK values of 5.5 and 9 for the Co(II)-requiring enzyme refer to individual groups on the enzyme. The value for k_{cat}/K_m of $3 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ would then be raised to the unrealistically high value of about $10^{11} \text{ M}^{-1} \cdot \text{s}^{-1}$. Hence, if either pK (5.5 or 9) refers to metal-bound water, it must be the lower one.

The co-ordination number of Zn(II) in β -lactamase II may well be greater than four in enzyme-substrate complexes, since in the free enzyme four residues bind the Zn(II) (Baldwin *et al.*, 1980a; Galdes *et al.*, 1980; Hill *et al.*, 1980).

Pseudomonas β -lactamase

The plot of k_{cat} against pH for the hydrolysis of cephalosporin C catalysed by the *Pseudomonas* β -lactamase (Fig. 4) again departs from a bell-shaped curve; this was also found for benzylpenicillin. The 'tail' is on the acid side. Fitting the curve gave the parameters listed in Table 2. The interpretation is based on Scheme 1, in which two ionic forms of an intermediate give product; the proton-poor form reacts about twice as fast. The

Table 1. Kinetic parameters and ionization constants for the hydrolysis of benzylpenicillin catalysed by β -lactamase II

The values were obtained by fitting the data to eqn. (1) and eqn. (2) by non-linear least-square methods described in the Materials and methods section.

	Zn(II)-requiring β -lactamase II		Co(II)- requiring β -lactamase II
	30°C	0°C	30°C
pH-dependence of $\tilde{k}_{\text{cat}}$			
$\text{p}K'_1$	5.1, 5.25*	5.6	5.8
$\text{p}K'_2$	8.0, 8.2*	8.3	8.4
$\text{p}K'_3$	10.2, 9.8*	9.9	10.3
$10^{-3} \times k_{+2} \text{ (min}^{-1}\text{)}$	53	4.38	8.77
$10^{-3} \times k'_{+2} \text{ (min}^{-1}\text{)}^\dagger$	14.6	1.09	2.69
k_{+2}/k'_{+2}	0.27	0.25	0.31
pH-dependence of $\tilde{k}_{\text{cat}}/\tilde{K}_m$			
$\text{p}K_1$	5.6		5.5
$\text{p}K_2$	7.6		9.0
$\tilde{k}_{\text{cat}}/\tilde{K}_m \text{ (}\mu\text{M}^{-1} \cdot \text{min}^{-1}\text{)}$	64		1820

* Values obtained from progress curves.

† k'_{+2} refers to the ionic form of the intermediate that has one fewer positive charges than the form that k_{+2} refers to (see Scheme 1).

Fig. 4. The pH-dependence of k_{cat} for the hydrolysis of cephalosporin C by the *Pseudomonas* β -lactamase. The rate of hydrolysis was measured in the pH-stat in 0.1 M-NaCl at 30°C. The curve is theoretical.

plot of k_{cat}/K_m was the usual bell-shaped curve (not shown) and fitting gave pK values of 5.2 and 9.7 (Table 2) probably arising from ionizations of the free enzyme. Ionization of the groups in the side chain of cephalosporin C seems to have at the most a very minor effect on the kinetics. The plot of k_{cat}/K_m for benzylpenicillin (Fig. 5) was a bell-shaped curve, fitted with pK values of 5.5 and 9.8. The pH-dependence of pK_m shows points of intersection of the extrapolated straight lines at pH values of 5.2–5.5 and 9.7–10.1 (Fig. 6); these arise from

Table 2. Kinetic parameters and ionization constants for the action of the *Pseudomonas* β -lactamase

The values for cephalosporin C were obtained by fitting the data to eqns. (1) and (2) by the methods described in the Materials and methods section. For benzylpenicillin, the values of k_{cat} were also obtained directly, and those of k_{cat}/K_m by the competing substrates method.

Substrate ...	Cephalosporin C	Benzylpenicillin
pH-dependence of k_{cat}		
pK'_1	4.7	4.2
pK'_2	7.4	8.3
pK'_3	10.1	10.7
$10^{-3} \times k_{+2}$ (min ⁻¹)*	9.3	4.2
$10^{-3} \times k'_{+2}$ (min ⁻¹)*	20.3	5.3
k_{+2}/k'_{+2}	2.2	1.3
pH-dependence of k_{cat}/K_m		
pK_1	5.2	5.5
pK_2	9.7	9.8
k_{cat}/K_m ($\mu M^{-1} \cdot min^{-1}$)	270	1795

* k_{+2} and k'_{+2} refer to the proton-rich and proton-poor forms respectively of the intermediate that forms product.

Fig. 5. The pH-dependence of k_{cat}/K_m for the hydrolysis of benzylpenicillin by the *Pseudomonas* β -lactamase. The values of k_{cat}/K_m for benzylpenicillin were deduced from the rate of hydrolysis of cephalosporin C measured spectrophotometrically in 0.1 M-NaCl at 30°C. The curve is theoretical.

ionizations in the free enzyme (Dixon & Webb, 1979). This method of treating the data thus reinforces the conclusions drawn from the pH-dependence of k_{cat}/K_m . On the other hand, the K_m for the hydrolysis of benzylpenicillin by a class A β -lactamase did not vary with pH (Waley, 1975). Both class A and class C β -lactamases have a pK of about 10, and have a lysine residue at position 73 or 83 in the sequence, where the active site serine is at position 70 or 80 respectively. Other possibilities are that the ionization is that of a tyrosine residue, or that there is a co-operative conformational transition involving more than one ionizing group.

Benzylpenicillin is a good substrate for the *Pseudomonas* β -lactamase, and the value of k_{cat}/K_m was about $3 \times 10^7 M^{-1} \cdot s^{-1}$. The same value was obtained for β -lactamase I with benzylpenicillin as substrate (Waley, 1975), and for Co(II)-requiring β -lactamase II with benzylpenicillin as substrate.

Previous work on the hydrolysis (and alcoholysis) of benzylpenicillin by the *Pseudomonas* β -lactamase has shown that an intermediate, the acyl-enzyme, accumulates at pH 5.5 and at pH 7 (Knott-Hunziker *et al.*, 1982b). Thus the pK values derived from the pH-dependence of k_{cat} for this β -lactamase (Table 2) are attributable mainly (or entirely) to the acyl-enzyme. In the acyl-enzyme, the acyl group is bound to a serine residue; in the serine proteinases the active-site histidine has a pK of about 7, but the experiments described here give no

Fig. 6. The pH-dependence of pK_m for the hydrolysis of benzylpenicillin and cephalosporin C by the *Pseudomonas* β -lactamase

The rates of hydrolysis of benzylpenicillin (●) and cephalosporin C (○) were measured as described in the text. The curve is drawn so that the lines of unit slope and vertical slope intersect at points 0.3 unit from the curve.

sign of such a residue in either this β -lactamase or β -lactamase II. The same was true for the class A β -lactamase studied previously (Waley, 1975). There is a need for further work on the nature of the groups that collaborate with the serine residue at the active site of class A and class C β -lactamases.

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