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A survey of the kinetic parameters of class C β-lactamases

Penicillins

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The interaction between six class C β-lactamases and various penicillins has been studied. All the enzymes behaved in a very uniform manner. Benzylpenicillin exhibited relatively low $k_{cat.}$ values (14–75 s^{-1}) but low values of K_m resulted in high catalytic efficiencies [$k_{cat.}/K_m = 10 \times 10^6$–$75 \times 10^6$ $M^{-1} \cdot s^{-1}$]. The $k_{cat.}$ values for ampicillin were 10–100-fold lower. Carbenicillin, oxacillin, cloxacillin and methicillin were very poor substrates, exhibiting $k_{cat.}$ values between 1×10^{-3} and 0.1 s^{-1}. The K_m values were correspondingly small. It could safely be hypothesized that, with all the tested substrates, deacylation was rate-limiting, resulting in acyl-enzyme accumulation.

INTRODUCTION

Jaurin & Grundström (1981) have previously determined the sequence of the *ampC* gene of *Escherichia coli* K12 and deduced the amino acid sequence of the corresponding protein, a β-lactamase of M_r 39600. On the basis of the primary structure, and of the catalytic mechanisms, two classes, A and B, of β-lactamases had already been identified by Ambler (1980). The protein encoded by the *ampC* gene clearly represented a new class of β-lactamases and became the founding member of the new class C.

Thereafter, various chromosome-encoded β-lactamases of Gram-negative bacteria were recognized as belonging to class C: all were periplasmic, considered to be cephalosporinases and produced by Enterobacteriaceae or related species: *Enterobacter cloacae* (Joris *et al.*, 1984), *Citrobacter freundii* (Lindberg & Normark, 1986), *Serratia marcescens* (Joris *et al.*, 1986) and *Pseudomonas aeruginosa* (Knott-Hunziker *et al.*, 1982).

In addition to the *Escherichia coli* enzyme, the complete primary structure, deduced from the sequence of the gene, is now known for one *Citrobacter* (Lindberg & Normark, 1986) and three *Enterobacter cloacae* (Galleni *et al.*, 1988*a*) enzymes. More than 70% of identities were found between any pair of the five proteins. In addition, sequences around the active-site serine residue have been determined for *Pseudomonas aeruginosa* (Knott-Hunziker *et al.*, 1982) and *Serratia marcescens* (Joris *et al.*, 1986) class C β-lactamases. Out of 14 residues nine are identical in the seven proteins, and for three others the replacements involve very similar side chains (valine, isoleucine and leucine). All M_r values are in the range 37000–40000. Inactivation by β-iodopenicillanic acid was characterized by a second-order rate constant (100–400 $M^{-1} \cdot s^{-1}$) distinctly lower than that observed with class A enzymes (2000–200000 $M^{-1} \cdot s^{-1}$), and branched pathways where hydrolysis of the inactivator also occurred were never observed (De Meester *et al.*, 1986). Many data can be found in the literature giving values for the kinetic constants of various enzyme–substrate interactions. However, those data were obtained under various experimental conditions and are difficult to use in rigorous comparisons between the enzymes. In addition, some very low activities have often been reported as 'zero'. If one realizes that the periplasmic concentrations of class C β-lactamases can be as high as 1 mM (Vu & Nikaido, 1985; Bush *et al.*, 1985), it is easy to understand that $k_{cat.}$ values as low as 10^{-2} s^{-1} have a non-negligible influence in determining the resistance of a super-producing strain.

We have undertaken site-directed-mutagenesis experiments in the hope of better understanding the mechanism of action of the class-C enzymes and of explaining the different behaviours of class A β-lactamases and of penicillin-sensitive enzymes. To do so it was necessary to measure the kinetic constants under strictly identical conditions. These data will also supply information about the uniformity of the properties of class C enzymes and about the correlation between the sequence-determined classification and that based on the substrate profile (Richmond & Sykes, 1973). Indeed, members of class C apparently correspond to types Ia, Ib and Id of the substrate-profile-based classification.

In the present paper we study various penicillin derivatives, and in the following paper (Galleni *et al.*, 1988*b*) cephalosporins and other β-lactams. Benzylpenicillin was used as the reference penicillin. The influence of an amino group (ampicillin) or of a carboxy group (carbenicillin) on the side chain was then examined. The other penicillins (oxacillin, cloxacillin and methicillin) had large, sterically hindered, side chains.

MATERIALS AND METHODS

Enzymes

The β-lactamases of *Serratia marcescens* SC8247 and of *Enterobacter cloacae* 908R and P99 were purified as described by Joris *et al.* (1986) and Joris *et al.* (1985) respectively. The β-lactamases of *Escherichia coli* K12 and *Citrobacter freundii* OS60 were gifts from F. Lindberg (University of Umeå, Umeå, Sweden) and that of *Pseudomonas aeruginosa* 18SH was a gift from R. Charnas (Hoffmann–La Roche, Basel, Switzerland).

β-Lactam antibiotics

Benzylpenicillin was from Rhône Poulenc (Paris, France), ampicillin and oxacillin were from Bristol Benelux S.A. (Brussels, Belgium), carbenicillin, cloxa-

cillin and methicillin were from Beecham Research Laboratories (Brentford, Middx., U.K.) and nitrocefin was from Glaxo Group Research (Greenford, Middx., U.K.).

Buffer

All experiments were performed in 10 mM-Hepes buffer, pH 8.2, containing 0.2 M-NaCl and 50 μg of bovine serum albumin/ml. Stock solutions of enzyme (0.5–1.0 mg/ml) were also diluted in the same buffer. The presence of bovine serum albumin stabilized the enzymes when diluted to low concentrations (< 50 μg/ml). Diluted samples of enzymes were not conserved for more than 10 h. The chosen pH was the optimum pH for class C β-lactamases (Bicknell *et al.*, 1983; Joris *et al.*, 1986).

Spectrophotometric measurements

All spectrophotometric measurements were performed with the help of a Beckman DU8 spectrophotometer interfaced to an Apple II microcomputer. The total sample volume was 480 μl.

Kinetic parameters for good substrates

Both benzylpenicillin and ampicillin exhibited rather low K_m values with all the enzymes (< 5 μM). Therefore, the $k_{cat.}$ values were measured with 0.5 and 1.0 mM concentrations of substrate. The absorbance at 230 nm decreased linearly until the reaction was virtually complete. The K_m values were measured as K_i values using 100 μM-nitrocefin as substrate under initial-rate conditions.

Parameters for poor substrates

Oxacillin, cloxacillin and carbenicillin were very poor substrates for all the enzymes. The values of K_m were extremely low (< 10 nM). The $k_{cat.}$ values were determined directly by using a 100 μM substrate concentration and monitoring the linear decrease of A_{230}. In other experiments the compounds were used as inactivators, with 100 μM-nitrocefin as the reporter substrate. The kinetic parameters for the interaction between nitrocefin and the six enzymes are determined in the following paper (Galleni *et al.*, 1988*b*). Over the time scale used in the present study (a few minutes) no substrate-induced inactivation was observed during hydrolysis of nitrocefin by any of the enzymes. In the presence of the poor substrates the rate of hydrolysis of nitrocefin progressively decreased until a steady state was reached. The curves were analysed with the help of the microcomputer as described by De Meester *et al.* (1987). This yielded the value of the pseudo-first-order rate constant for the inactivation, k_i. On the basis of the simple model:

$$E+C \overset{K}{\rightleftharpoons} EC \overset{k_2}{\rightarrow} EC^* \overset{k_3}{\rightarrow} E+P$$

(where EC* is the acyl-enzyme intermediate), which is generally accepted for the interaction between β-lactamases and their substrates, the value of k_i is given by eqn. 1:

$$k_i = k_3 + k_f = k_3 + \frac{k_2[C]}{[C] + K\frac{K_m^S + [S]}{K_m^S}} \quad (1)$$

where S is the reporter substrate and K_m^S the corresponding Henri–Michaelis constant. The rate of hydrolysis of the reporter substrate at the steady state (v_{ss}) was also compared with that observed in the absence of inactivator (v_0):

$$\frac{v_0}{v_{ss}} = 1 + \frac{K_m^S}{[S]+K_m^S} \cdot \frac{[C]}{K} \cdot \frac{k_2+k_3}{k_3} \quad (2)$$

A linear variation of k_i plotted versus [C] indicated that [C] was $\ll K\{(K_m^S+[S])/K_m^S\}$. In this case k_3 could be obtained by extrapolation to [C] = 0 and k_2/K from the slope of the line (eqn. 1), and the highest observed value of k_i supplied a minimum value for k_2. When this minimum value was $\gg k_3$, a second estimate of k_3 could easily be obtained from eqn. (2).

With methicillin, the value of $k_{cat.}$ was determined at a saturating concentration (0.5–1.0 mM) by monitoring the decrease of A_{230} and that of K_m was measured as a K_i value with nitrocefin as substrate.

RESULTS AND DISCUSSION

As shown by Table 1, the $k_{cat.}$ values for benzylpenicillin were relatively low (14–76 s^{-1}) when compared with those observed with good cephalosporin substrates (see Galleni *et al.*, 1988*b*). Conversely, the K_m values were also very low, which resulted in very high $k_{cat.}/K_m$ values (10×10^6–75×10^6 $M^{-1}\cdot s^{-1}$). On the basis of the latter criterion and as already noted before (Bicknell *et al.*, 1983; Joris *et al.*, 1986), benzylpenicillin is one of the best substrates for the class C β-lactamases, an observation that somewhat belies their classification as 'cephalosporinases'.

The $k_{cat.}$ values for ampicillin were one order of magnitude lower. Since the K_m values were not very different, the $k_{cat.}/K_m$ values were also an order magnitude smaller. Our values were in good agreement with those reported by Nikaido & Normark (1987) for *Escherichia coli* K12, by Furth (1979) for *Pseudomonas aeruginosa*, by Tajima (1980) for *Citrobacter freundii* and by Cartwright & Waley (1983) for *Enterobacter cloacae* P99 and *Pseudomonas aeruginosa* with the exception of the K_m of the *Pseudomonas aeruginosa* β-lactamase for ampicillin, for which Cartwright & Waley (1983) reported a value of 150 μM.

As generally accepted, cloxacillin (Table 2), oxacillin (Table 3) and carbenicillin (Table 4) were very poor substrates characterized by low values of k_3 (1×10^{-3}–40×10^{-3} s^{-1}). However, the values of k_2/K were always higher than 2×10^5 $M^{-1}\cdot s^{-1}$ and sometimes reached 10×10^6 $M^{-1}\cdot s^{-1}$, which indicated a very efficient formation of the acyl-enzyme. Consequently, the compounds behaved as transient inactivators of the enzymes.

With the *Enterobacter cloacae* P99 and the *Pseudomonas aeruginosa* enzymes methicillin (50 and 100 μM) induced biphasic kinetics. The size of the burst was significantly higher than the enzyme concentration, indicating a phenomenon similar to that observed by Citri *et al.* (1976). The values of $k_{cat.}$ and K_m shown in Table 5 were measured after establishment of the steady-state characteristic of the linear second phase.

With the *Citrobacter* and the *Escherichia coli* K12 enzymes the first phase was not detected. However, it was not unlikely that the same phenomenon occurred, but at a somewhat higher rate and with a smaller decrease in the reaction rate. When nitrocefin was used for the measurement of the K_i values a progressive

Table 1. Kinetic parameters for benzylpenicillin and ampicillin

All K_m values were determined as K_i values. The values for the interaction between the *S. marcescens* β-lactamase and benzylpenicillin are from Joris *et al.* (1986) (pH 8.0, 0.2 M-NaCl, 0.2 mg of bovine serum albumin/ml).

Source of enzyme	Benzylpenicillin			Ampicillin		
	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-6} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-6} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)
Enterobacter cloacae P99	0.6±0.1	14±1.5	23±5	0.4±0.05	0.74±0.05	1.8±0.4
Enterobacter cloacae 908R	0.5±0.1	18±0.7	36±8	0.4±0.03	0.53±0.06	1.3±0.3
Citrobacter freundii	0.4±0.05	31±3	75±15	0.2±0.01	6.5±0.7	30±5
Escherichia coli K12	4.4±0.1	45±6	10±1	3.5±0.03	4.2±0.3	1.2±0.2
Serratia marcescens	1.7±0.2	75±3	44±6	0.01±0.002	0.46±0.04	46±14
Pseudomonas aeruginosa	1.7±0.3	76±7	45±6	0.5±0.03	4.4±0.5	9±1.5

Table 2. Kinetic parameters for cloxacillin

$(k_3)_{extrap.}$: from the extrapolation for [C] = 0 (eqn. 1). $(k_3)_{ss}$: from the steady-state value (eqn. 2). $(K_m)_{calc.}$: from the values of k_2/K and k_3 ($K_m = k_3 K/k_2$). Abbreviation: N.D., not determined.

Source of enzyme	$10^{-6} \times k_2/K$ ($M^{-1} \cdot s^{-1}$)	$10^3 \times (k_3)_{extrap.}$ (s^{-1})	$10^3 \times (k_3)_{ss}$ (s^{-1})	$10^3 \times k_{cat.}$ (s^{-1})	$(K_m)_{mes.}$ (nM)	$(K_m)_{calc.}$ (nM)
Enterobacter cloacae P99	11±0.7	N.D.	3±0.6	6±0.6	N.D.	0.4±0.15
Enterobacter cloacae 908R	9±0.5	5.8	3±0.3	4±0.5	N.D.	0.5±0.2
Citrobacter freundii	11±1	10–18	6±0.7	N.D.	N.D.	5–10
Escherichia coli K12	7±0.8	N.D.	3±0.5	N.D.	N.D.	0.5±0.15
*Pseudomonas aeruginosa**	[1±0.3 (calc.)]	N.D.	N.D.	10±2	11±1.5	N.D.

* Inactivation was too fast to be studied. $k_{cat.}$ was obtained from the linear decrease of A_{230} with 100 μM-cloxacillin; K_m was measured as a K_i value and k_2/K was computed as $k_{cat.}/K_m$.

Table 3. Kinetic parameters for oxacillin

The value in parenthesis was not very reliable since the extrapolated value was much smaller than the smallest measured k_i value. Abbreviation: N.D., not determined.

Source of enzyme	$10^{-6} \times k_2/K$ ($M^{-1} \cdot s^{-1}$)	$10^3 \times (k_3)_{extrap.}$ (s^{-1})	$10^3 \times (k_3)_{ss}$ (s^{-1})	$10^3 \times k_{cat.}$ (s^{-1})	$(K_m)_{mes.}$ (nM)	$(K_m)_{calc.}$ (nM)
Enterobacter cloacae P99	7±0.5	3.8	3±0.5	6±1	N.D.	0.4–0.8
Enterobacter cloacae 908R	7±1	(2)	4±0.5	8±0.5	N.D.	0.6–1.1
Citrobacter freundii	12±1	14	5±0.2	N.D.	N.D.	0.4–1
Escherichia coli K12	N.D.	N.D.	N.D.	N.D.	8±2	N.D.
*Pseudomonas aeruginosa**	[1.6±0.3 (calc.)]	N.D.	N.D.	40±3	24±3	N.D.

* For the *Pseudomonas* enzyme see the footnote of Table 2.

Table 4. Kinetic parameters for carbenicillin

Abbreviation: N.D., not determined.

Source of enzyme	$10^{-5} \times k_2/K$ ($M^{-1} \cdot s^{-1}$)	$10^3 \times (k_3)_{extrap.}$ (s^{-1})	$10^3 \times (k_3)_{ss}$ (s^{-1})	$10^3 \times k_{cat.}$ (s^{-1})	$(K_m)_{calc.}$ (nM)
Enterobacter cloacae P99	2.6±0.3	N.D.	1.3±0.3	3±0.4	5–11
Enterobacter cloacae 908R	3.7±0.5	4	1.3±0.2	4±0.6	4–11
Citrobacter freundii	70±8	1	2±0.3	N.D.	0.15–0.3
Escherichia coli K12	2.3±0.1	9	4±0.3	N.D.	18–39
Pseudomonas aeruginosa	2.4±0.5	3	5±1	3±2	13–20

Table 5. Kinetic parameters for methicillin

Source of enzyme	$10^2 \times k_{cat.}$ (s^{-1})	$(K_m)_{app.}$† (nM)	$10^{-5} \times k_{cat.}/K_m$
Enterobacter cloacae P99	0.7±0.1*	30±4	3±1
Citrobacter freundii	1±0.1	2±0.3	50±10
Escherichia coli K12	8±0.2	150±30	5±1
Pseudomonas aeruginosa	2±0.4*	29±20	7±5

* Computed from the linear hydrolysis observed during the second phase.

† Measured as a K_i value after establishment of the final steady state in the case of *Enterobacter cloacae* P99 and *Pseudomonas aeruginosa*.

slowdown of the reaction followed by a steady state was also observed for the P99 enzyme.

The data collected in our study clearly indicated a high degree of similarity between all examined class C enzymes. The two *Enterobacter* enzymes were particularly similar, and it seemed that the four observed differences in the primary structure had very little influence on the kinetic parameters. In fact, the two enzymes were nearly indistinguishable, the only detectable effects of the mutations appearing to be on the surface properties of the proteins, far from the active sites but influencing the characteristics of the crystals, which belonged to different space groups (P. Charlier & O. Dideberg, unpublished work).

As underlined before, benzylpenicillin exhibited in all cases relatively low $k_{cat.}$ values, but the very low K_m values resulted in high catalytic efficiencies ($k_{cat.}/K_m$). The low K_m values could be attributed to a low k_3/k_2 ratio, in agreement with the results obtained by Knott-Hunziker *et al.* (1982), who showed an accumulation of acyl-enzyme with benzylpenicillin and the *Pseudomonas aeruginosa* β-lactamase. Accumulation of acyl-enzyme was also likely in the interaction with ampicillin and carbenicillin, where low K_m values were observed. The addition of an amino group on the side chain of benzylpenicillin resulted in a 10–100-fold decrease of $k_{cat.}$ and that of a carboxylate in a 10000-fold decrease of $k_{cat.}$. It will be interesting to see how the three-dimensional structure of the binding site explains this enormous effect of a carboxylate group! This latter result clearly distinguished the class C from the class A enzymes, for which carbenicillin is usually a reasonably good substrate (see, e.g., Labia *et al.*, 1979). With carbenicillin, cloxacillin and oxacillin accumulation of the acyl-enzyme could be directly monitored thanks to the very low values of k_3. Again, and in spite of the substrate-induced inactivation observed with some class A enzymes and cloxacillin or oxacillin, the $k_{cat.}$ values observed with the class C β-lactamases were much lower than with class A. Finally, with methicillin substrate-induced inactivation was observed in some cases and the rates of the reactions after the transition were much lower than those observed with class-A enzymes.

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In the following paper (Galleni *et al.*, 1988*b*) we present a study of other substrates, which supplies further information relevant to the present discussion.

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