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

Cephalosporins and other β -lactam compounds

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Various cephalosporins, cefoxitin, moxalactam, imipenem and aztreonam were studied as substrates of six class C β -lactamases. Nitrocefin, cephaloridine, cefazolin, cephalothin and cephalixin were good substrates, with k_{cat} values ranging from 27 to 5000 s⁻¹. Cefuroxime, cefotaxime and cefoxitin exhibited low k_{cat} values (0.010–1.7 s⁻¹) and low K_m values, which suggested a rate-limiting deacylation. Imipenem and aztreonam were even poorer substrates (k_{cat} 2×10^{-4} – 3×10^{-2} s⁻¹) and, in the presence of a reporter substrate, behaved as transient inactivators. With moxalactam, biphasic kinetics were observed, indicating a possible rearrangement of the acyl-enzyme.

INTRODUCTION

In the preceding paper we described the interactions between various class C β -lactamases and a series of penicillins (Galleni & Frère, 1988). In the present paper we present data concerning the interactions between the same enzymes and various cephalosporins, a cephamycin (cefoxitin), an oxo-cephamycin (moxalactam), a carbapenem (imipenem or *N*-formimidoyl-thienamycin) and monobactams (aztreonam and camuronam). The compounds were chosen as representatives of various classes of potential β -lactamase substrates. Among the cephalosporins, cephaloridine, cefazolin, cephalothin and cephalixin are of the first generation and cefuroxime and cefotaxime of the second and third generations respectively. Cephaloridine and cephalixin were the cephalosporin substrates used by Richmond & Sykes (1973) in their classification of the β -lactamases based on kinetic properties. Nitrocefin, the chromogenic substrate widely utilized in detailed kinetic studies (De Meester *et al.*, 1986, 1987), and PADAC, another chromogenic substrate, were also included in the survey.

MATERIALS AND METHODS

Materials

The enzymes were those described in the preceding paper (Galleni & Frère, 1988). PADAC was purchased from Calbiochem (Behring Diagnostics, La Jolla, CA, U.S.A.). Nitrocefin and cefuroxime were from Glaxo Group Research (Greenford, Middx., U.K.), aztreonam was from the Squibb Institute for Medical Research (Princeton, NJ, U.S.A.), imipenem and cefoxitin were from Merck, Sharp and Dohme Research Laboratories (Rahway, NJ, U.S.A.) cefotaxime was from Hoechst-Roussel (Romainville, France), camuronam was from Hoffmann-La Roche (Basel, Switzerland) and cephaloridine, cefazolin, cephalothin, cephalixin and moxalactam

were from Eli Lilly and Co. (Indianapolis, IN, U.S.A.). All of these compounds were kindly given by the respective companies.

Kinetic parameters for good substrates

The values of k_{cat} and K_m were measured by analysing the complete progress curves of the reaction with the help of a Beckman DU8 spectrophotometer interfaced to an Apple II microcomputer (Joris *et al.*, 1985; De Meester *et al.*, 1987). The enzyme was added to 450 μ l of substrate solution in 10 mM-Hepes buffer, pH 8.2, containing 0.2 M-NaCl and 50 μ g of bovine serum albumin/ml. The conditions were such that the reaction was 90% complete within 60–90 s. Table 1 summarizes the other experimental conditions. In some cases where low K_m values (approx. 10 μ M) were found, the values were checked in a separate experiment in which the tested compound was used as a competitive inhibitor versus 100 μ M-nitrocefin. With cephalixin all K_m values were measured as K_i , and k_{cat} values were obtained with saturating concentrations (100 and 200 μ M) of substrate. The absence of substrate-induced inactivation was demonstrated by modifying both substrate and enzyme initial concentrations. As discussed before (Joris *et al.*, 1986), that phenomenon results in a decrease of K_m and $V_{\text{max}}/[E]_0$ when the enzyme concentration is increased.

Poor substrates

Cefuroxime, cefotaxime and cefoxitin were poor substrates of all the enzymes (k_{cat} = 0.01–1.7 s⁻¹) and also exhibited very low K_m values. The k_{cat} values were determined with large quantities of enzyme by monitoring the linear decrease of absorbance at a saturating concentration of substrate. The K_m values were determined as K_i values as above. The other experimental conditions are summarized in Table 1.

Abbreviation used: PADAC, 7-(thien-2-ylacetamido)-3-[2-(4-*NN*-dimethylaminophenylazo)pyridinium methyl]-3-cephem-4-carboxylic acid.

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Table 1. Experimental conditions for the determination of kinetic parameters

Abbreviation: QE, quantity of enzyme. (a) For the measurement of k_{cat} ; (b) for the measurement of K_m ; (c) for the measurement of the inactivation rate.

Antibiotic	<i>Enterobacter cloacae</i> P99 enzyme		<i>Enterobacter cloacae</i> 908R enzyme		<i>Citrobacter freundii</i> enzyme		<i>Escherichia coli</i> K12 enzyme		<i>Pseudomonas aeruginosa</i> enzyme		<i>Serratia marcescens</i> enzyme	
	[S] (μ M)	QE (ng)	[S] (μ M)	QE (ng)	[S] (μ M)	QE (ng)	[S] (μ M)	QE (ng)	[S] (μ M)	QE (ng)	[S] (μ M)	QE (ng)
Nitrocefin	50-100	10-30	50-100	10-30	50-100	5-20	100-200	25-50	50-100	5-20	-	-
	30-100	30-100	30-100	30-100	30-100	70-100	30-100	100-150	30-100	30-50	-	-
PADAC	30-50	100-200	100-200	30-50	100-200	30-100	100-200	30-100	100-200	30-50	-	-
Cephaloridine	50-100	50-100	50-100	50-100	50-100	30-50	50-100	50-100	50-100	50-100	-	-
Cephalothin	1000-2000	50-100	1000-2000	50-100	1000-2000	50-100	1000-2000	50-100	1000-2000	50-100	-	-
Cefazolin	100-200	100-200	100-200	100-200	100-200	100-200	100-200	100-200	100-200	100-200	100-200	100-200
Cephalexin (a)	8-43	6	10-43	6	10-50	6	10-43	6	10-43	6	100-200	100-200
(b)	100-75	10000	100-75	10000	100-75	10000	100-75	4300	100-75	7000	80-400	6
Cefuroxime (a)	0.02-0.2	3-5	0.01-0.2	3-5	0.1-2	5-10	0.04-2	2-5	0.02-0.1	3-5	-	-
(b)	100-200	20000	100-200	20000	100-200	10000	100-200	10000	100-200	14000	-	-
Cefotaxime (a)	0.02-2	5-10	0.02-2	5-10	0.4-2	3-5	1-4	2-5	1-4	3-10	-	-
(b)	100-150	10000	100-150	10000	100-150	7800	100-150	8600	100-150	7000	-	-
Cefoxitin (a)	0.04-0.2	5-10	0.04-0.2	5-10	0.2-1	3-5	0.2-2	2-5	-	-	-	-
(b)	20-60	50	20-60	50	20-60	50	20-60	50	20-60	50	20-60	50
Aztreonam (a)	0.3-0.5	5-10	0.3-0.9	5-10	1-2	2-10	0.02-0.05	2-5	1.5-3.5	5-10	10-17	20-40
(c)	1-3	5-10	1-2	5-10	1-3	2-10	3-5	2-5	1-3	5-10	1-3	20-40
Imipenem (c)	100-200	40000	-	-	-	-	100-200	28000	100-200	28000	-	-
Moxalactam												

Table 2. Kinetic parameters for good cephalosporin substrates (I)

Source of enzyme	Nitrocefin			Cephaloridine			Cefazolin		
	k_{cat} (s^{-1})	K_m (μ M)	$10^{-6} \times k_{cat}/K_m$ ($M^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μ M)	$10^{-6} \times k_{cat}/K_m$ ($M^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μ M)	$10^{-6} \times k_{cat}/K_m$ ($M^{-1} \cdot s^{-1}$)
<i>Enterobacter cloacae</i> P99	780 $\pm$ 30	25 $\pm$ 1	31 $\pm$ 2	700 $\pm$ 20	70 $\pm$ 4	10 $\pm$ 0.8	3000 $\pm$ 80	1500 $\pm$ 200	2 $\pm$ 0.3
<i>Enterobacter cloacae</i> 908R	780 $\pm$ 30	23 $\pm$ 2	34 $\pm$ 2	600 $\pm$ 10	75 $\pm$ 4	8 $\pm$ 0.2	3000 $\pm$ 200	1500 $\pm$ 400	2 $\pm$ 0.6
<i>Citrobacter freundii</i>	330 $\pm$ 20	12 $\pm$ 1	28 $\pm$ 1	700 $\pm$ 30	35 $\pm$ 3	20 $\pm$ 2	5000 $\pm$ 500	600 $\pm$ 200	8 $\pm$ 0.6
<i>Escherichia coli</i> K12	490 $\pm$ 90	500 $\pm$ 100	1 $\pm$ 0.2	130 $\pm$ 13	170 $\pm$ 3	13 $\pm$ 0.2	150 $\pm$ 9	400 $\pm$ 30	0.4 $\pm$ 0.02
<i>Serratia marcescens</i> *	1240 $\pm$ 60	40 $\pm$ 2	30 $\pm$ 3	1100 $\pm$ 50	275 $\pm$ 25	4 $\pm$ 0.5	1300 $\pm$ 100	540 $\pm$ 4.5	2.4 $\pm$ 0.4
<i>Pseudomonas aeruginosa</i>	742 $\pm$ 30	27 $\pm$ 2	27 $\pm$ 2	110 $\pm$ 17	20 $\pm$ 1	5 $\pm$ 0.6	← Biphasic →		

* Data taken from Joris *et al.* (1986).

Table 3. Kinetic parameters for good cephalosporin substrates (II)

The data for the interaction between cephalothin and the *Serratia marcescens* β -lactamase are from Joris *et al.* (1986). Those values were obtained before the substrate-induced inactivation became significant.

Source of enzyme	Cephalothin			Cephalexin		
	$k_{\text{cat.}}$ (s^{-1})	K_m (μM)	$10^{-6} \times$ $k_{\text{cat.}}/K_m$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)	$k_{\text{cat.}}$ (s^{-1})	$K_m^\dagger$ (μM)	$10^{-6} \times$ $k_{\text{cat.}}/K_m$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)
<i>Enterobacter cloacae</i> P99	200 $\pm$ 8	9 $\pm$ 0.7*	20 $\pm$ 1	70 $\pm$ 5	10 $\pm$ 1	7 $\pm$ 1
<i>Enterobacter cloacae</i> 908R	180 $\pm$ 16	9 $\pm$ 1*	20 $\pm$ 3	72 $\pm$ 4	8.5 $\pm$ 0.6	8.5 $\pm$ 1
<i>Citrobacter freundii</i>	210 $\pm$ 9	13 $\pm$ 1	16 $\pm$ 2	150 $\pm$ 10	12 $\pm$ 1	12.5 $\pm$ 2
<i>Escherichia coli</i> K12	300 $\pm$ 15	42 $\pm$ 2	7 $\pm$ 1	38 $\pm$ 3	4 $\pm$ 0.4	9.5 $\pm$ 1.7
<i>Serratia marcescens</i>	1100 $\pm$ 50	67 $\pm$ 4	16 $\pm$ 1.7	80 $\pm$ 7	30 $\pm$ 5	2.7 $\pm$ 0.7
<i>Pseudomonas aeruginosa</i>	430 $\pm$ 30	17 $\pm$ 0.4	25 $\pm$ 2	27 $\pm$ 3	3 $\pm$ 0.1	9 $\pm$ 1.3

* Also measured as K_i values.
† Only measured as K_i values.

Imipenem and aztreonam

The values of $k_{\text{cat.}}$ were extremely low (2×10^{-4} – $3 \times 10^{-2} \text{ s}^{-1}$). The kinetic parameters for imipenem were derived as explained in the preceding paper (Galleni & Frère, 1988) for oxacillin, cloxacillin and carbenicillin. For aztreonam the values of k_2/K and $(k_3)_{\text{ss}}$ were similarly measured, but the value of k_3 was also obtained by measuring the rate of recovery of the enzymic activity after titration of concentrated enzyme (1 mg/ml) to negligible activity and diluting a sample 1000-fold in a 100 μM solution of nitrocefin.

Moxalactam

Biphasic kinetics were observed. A value of $k_{\text{cat.}}$ was measured for the second, linear, phase with 100 and 200 μM -moxalactam and 28–40 μg of enzyme/ml. For the P99 enzyme a value of K_i was also computed during the linear phase with 100 μM -nitrocefin as substrate.

RESULTS AND DISCUSSION

Good substrates

PADAC was a good substrate. At a 100 μM concentration it was completely hydrolysed as rapidly as cephaloridine by all the enzymes. However, in all cases biphasic progress curves were observed, making an analysis impossible on the basis of a simple model. Biphasic kinetics were also observed in the present study with cefazolin and the *Pseudomonas aeruginosa* β -lactamase and in previous work with cephalothin and the *Serratia marcescens* β -lactamase (Joris *et al.*, 1986). Nitrocefin, cephaloridine, cefazolin, cephalexin and cephalothin were good substrates of all the enzymes (Tables 2 and 3) with $k_{\text{cat.}}$ values ranging from 27 to 5000 s^{-1} . However, the K_m values were also higher than those observed with benzylpenicillin, so that this latter substrate exhibited the highest $k_{\text{cat.}}/K_m$ value for the *Citrobacter freundii*, *Serratia marcescens* and *Pseudomonas aeruginosa* enzymes. For the *Enterobacter cloacae* and *Escherichia coli* enzymes the highest $k_{\text{cat.}}/K_m$ ratios were observed with nitrocefin and cephaloridin respectively.

Poor substrates

Table 4 shows the measured values and the calculated values of $k_{\text{cat.}}/K_m (=k_2/K)$. The second-order rate constants characteristic of the formation of the acyl-enzyme are often rather high ($\geq 1 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$). The low K_m values suggested that deacylation was the rate-limiting step and that, in consequence, $k_{\text{cat.}} = k_3$.

Imipenem and aztreonam

Both compounds practically behaved as rather efficient transient inactivators (Table 5). The values of k_3 were very low for imipenem (2×10^{-3} – $16 \times 10^{-3} \text{ s}^{-1}$) and even lower (2×10^{-4} – $40 \times 10^{-4} \text{ s}^{-1}$) for aztreonam. The values of k_2/K were still high (10 000–300 000 $\text{M}^{-1} \cdot \text{s}^{-1}$). However, one should remember that some strains constitutively produce extremely large periplasmic concentrations of class C enzymes (up to 1 mM). Even with a $k_{\text{cat.}}$ value of 10^{-3} s^{-1} , such situations can result in a rather efficient hydrolysis of the β -lactam ($V_{\text{max.}} = k_{\text{cat.}} \times [\text{E}]_0 = 1 \mu\text{M} \cdot \text{s}^{-1}$), and if the penetration of the antibiotic is hindered by the outer membrane, a large proportion of the entering β -lactam can be destroyed (Frère *et al.*, 1988). The values of k_2/K and k_3 were also measured for camuronam, another monobactam, with the P99 and *Pseudomonas* enzymes. The results were not very different from those obtained with aztreonam:

P99,

$$k_2/K = 40 \times 10^3 \pm 5 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1};$$

$$k_3 = 1 \times 10^{-3} \pm 0.2 \times 10^{-3} \text{ s}^{-1};$$

Pseudomonas,

$$k_2/K = 4 \times 10^3 \pm 0.5 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1};$$

$$k_3 = 3.6 \times 10^{-3} \pm 0.5 \times 10^{-3} \text{ s}^{-1}.$$

Moxalactam

Fig. 1 shows a time course of the hydrolysis of moxalactam by the *Enterobacter cloacae* P99 β -lactamase. A progressive decrease of the reaction rate, leading to a final steady state, was clearly detectable. The height of the burst corresponded to a concentration of 10 μM , significantly larger than the enzyme concentration (1.1 μM). The burst could thus not be attributed to the accumulation of the acyl-enzyme and it was likely that a

Table 4. Kinetic parameters for poor cephalosporin substrates

The K_m values were obtained as K_i values.

Source of enzyme	Cefuroxime			Cefotaxime			Cefoxitin		
	k_{cat} (s^{-1})	K_m (μM)	$10^{-6} \times k_{cat}/K_m$ ($M^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	$10^{-6} \times k_{cat}/K_m$ ($M^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	$10^{-6} \times k_{cat}/K_m$ ($M^{-1} \cdot s^{-1}$)
<i>Enterobacter cloacae</i> P99	0.05 $\pm$ 0.006	0.016 $\pm$ 0.003	3.1 $\pm$ 0.35	0.015 $\pm$ 0.004	0.010 $\pm$ 0.004	1.5 $\pm$ 0.16	0.06 $\pm$ 0.005	0.024 $\pm$ 0.002	2.5 $\pm$ 0.1
<i>Enterobacter cloacae</i> 908R	0.05 $\pm$ 0.002	0.016 $\pm$ 0.001	3.1 $\pm$ 0.1	0.010 $\pm$ 0.003	0.010 $\pm$ 0.0007	1 $\pm$ 0.2	0.08 $\pm$ 0.009	0.020 $\pm$ 0.003	4 $\pm$ 0.3
<i>Citrobacter freundii</i>	0.04 $\pm$ 0.007	0.020 $\pm$ 0.002	2 $\pm$ 0.3	0.017 $\pm$ 0.002	0.005 $\pm$ 0.001	3.4 $\pm$ 1	0.32 $\pm$ 0.04	0.250 $\pm$ 0.02	1.3 $\pm$ 0.09
<i>Escherichia coli</i> K12	0.15 $\pm$ 0.04	0.150 $\pm$ 0.02	1 $\pm$ 0.16	0.17 $\pm$ 0.04	1.7 $\pm$ 0.3	0.1 $\pm$ 0.001	0.2 $\pm$ 0.04	0.650 $\pm$ 0.70	0.3 $\pm$ 0.02
<i>Serratia marcescens</i> *	0.8 $\pm$ 0.15	0.6 $\pm$ 0.06	1.3 $\pm$ 0.4	1.7 $\pm$ 0.2	12 $\pm$ 3	0.14 $\pm$ 0.05	0.014 $\pm$ 0.002	0.3 $\pm$ 0.03	0.04 $\pm$ 0.01
<i>Pseudomonas aeruginosa</i>	0.05 $\pm$ 0.007	0.02 $\pm$ 0.003	2.5 $\pm$ 0.4	0.15 $\pm$ 0.03	0.2 $\pm$ 0.03	0.75 $\pm$ 0.01	0.12 $\pm$ 0.02	0.050 $\pm$ 0.004	2.4 $\pm$ 0.14

* The values are taken from Joris *et al.* (1986).

Table 5. Kinetic parameters for imipenem and aztreonam

$K_{m(cal.)}$ is the value of K_m computed as $(k_3/k_2)K_i$ (k_3) is the value of k_3 as measured in the re-activation experiment. (k_3) is the value of k_3 as obtained from the steady state in the presence of the reporter substrate. Abbreviation: N.D., not determined.

Source of enzyme	Imipenem			Aztreonam		
	$10^{-3} \times k_2/K$ ($M^{-1} \cdot s^{-1}$)	$10^3 \times (k_3)_{ss}$ (s^{-1})	$10^3 \times k_{cat}$ (s^{-1})	$10^{-3} \times k_2/K$ ($M^{-1} \cdot s^{-1}$)	$10^4 \times (k_3)_{ss}$ (s^{-1})	$10^4 \times K_{m(cal.)}$ (nm)
<i>Enterobacter cloacae</i> P99	60 $\pm$ 0.4	2 $\pm$ 0.2	3 $\pm$ 0.1	260 $\pm$ 20	4.4 $\pm$ 0.3	2 $\pm$ 0.3
<i>Enterobacter cloacae</i> 908R	145 $\pm$ 16	4.2 $\pm$ 0.6	3.1 $\pm$ 0.04	290 $\pm$ 20	3.5 $\pm$ 0.6	3 $\pm$ 0.5
<i>Citrobacter freundii</i>	140 $\pm$ 30	9 $\pm$ 1*	16 $\pm$ 0.7	180 $\pm$ 20	3.2 $\pm$ 0.4	2 $\pm$ 0.4
<i>Escherichia coli</i> K12	11.2 $\pm$ 1	8 $\pm$ 0.3	10 $\pm$ 0.9	135 $\pm$ 30	1.6 $\pm$ 0.3	N.D.
<i>Serratia marcescens</i>	27 $\pm$ 0.5	2.2 $\pm$ 0.3	1 $\pm$ 0.1	12 $\pm$ 1.7	7 $\pm$ 1	N.D.
<i>Pseudomonas aeruginosa</i>	88 $\pm$ 10	16 $\pm$ 0.3	30 $\pm$ 0.1	58 $\pm$ 10	23 $\pm$ 3	40 $\pm$ 10

* k_3 was also obtained by extrapolation of the line k_a versus $[C]$ for $[C] = 0$. A value of $10 \times 10^{-3} s^{-1}$ was found.

Fig. 1. Time course of the hydrolysis of moxalactam by the *Enterobacter cloacae* P99 β -lactamase

To 450 μ l of 100 μ M-moxalactam was added 20 μ g of enzyme (in 20 μ l). The arrows delineate the portion of the curve that was used to compute k_{cat} and the extrapolated (broken) line for measuring the size of the 'burst'.

Table 6. Moxalactam: parameters after establishment of the steady state

Abbreviation: N.D., not determined.

Source of enzyme	$10^3 \times k_{cat}$ (s^{-1})	K_i (nM)
<i>Enterobacter cloacae</i> P99	9 ± 1	70 ± 10
<i>Escherichia coli</i> K12	10 ± 1	N.D.
<i>Pseudomonas aeruginosa</i>	6 ± 1	N.D.

branched pathway occurred, similar to that described by Faraci & Pratt (1985) and involving the rearrangement of the oxo-cephalosporoyl moiety. Since our goal was not to study that type of phenomenon, we measured the value of k_{cat} by monitoring the hydrolysis of moxalactam and that of K_i by monitoring the hydrolysis of nitrocefin after the steady state was established (Table 6).

Comparison with previously published data

It would take too long to attempt to compare our results with all the kinetic parameters that have been previously published for the five different enzymes studied (the two *Enterobacter cloacae* enzymes can be considered as practically identical).

Table 7 gives a summary of a comparison with some published data. The major discrepancies are underlined. It is interesting to note the generally good agreement with the data of Tajima *et al.* (1980) and Nikaido & Normark (1987). With two exceptions, the values reported by Cartwright & Waley (1983) are also similar to ours.

Homogeneity of class C

The similarity of catalytic profiles that had been noticed for penicillin substrates in the preceding paper (Galleni & Frère, 1988) clearly extended to all the other substrates that had been chosen. This result can be correlated to the high percentage of conserved residues

Table 7. Comparison of our parameters with previously published data

The Table gives the ratio (published value)/(our value) for K_m and k_{cat} . The major discrepancies (factor of 10 or more) are underlined. N, not given by the authors. References: a, Cartwright & Waley (1983); b, Minami *et al.* (1980); c, Seeborg *et al.* (1983); d, Letarte *et al.* (1977); e, Bush *et al.* (1982); f, Furth (1979); g, Livermore (1983); h, Berks *et al.* (1982); i, Nikaido & Normark (1987); j, Tajima *et al.* (1980).

Source of enzyme	Cephaloridine			Cefazolin			Cephalothin			Cefotaxime			Cefuroxime			Cefoxitin		
	K_m ratio	k_{cat} ratio	Ref.	K_m ratio	k_{cat} ratio	Ref.	K_m ratio	k_{cat} ratio	Ref.	K_m ratio	k_{cat} ratio	Ref.	K_m ratio	k_{cat} ratio	Ref.	K_m ratio	k_{cat} ratio	Ref.
<i>Enterobacter cloacae</i>	N	1.2	a	0.5	0.04	b	6.7	1	a	5	N	b	2.5	N	a	1	N	a
	8	0.15	b				12	1.1	b	0.5	N	j	0.4	N	j	1	N	j
	2.5	N	c															
	2	0.25	d															
<i>Citrobacter freundii</i> <i>Escherichia coli</i>	7	N	e				0.7	1	j	0.5	N	j	0.4	N	j	1	N	j
	2	1.6	f	0.4	1.7	j	0.5	0.8	i	0.1	0.3	i				0.34	0.75	i
	1.4	1.2	i	3.8	6													
<i>Pseudomonas aeruginosa</i>	5	0.4	f				0.04	0.7	f	20	N	a		N	a	1	N	a
	5	0.5	a				0.04	0.7	a	15	N	h		N	h	3.4	N	h
	10	N	g															

Table 8. Comparison between reference values used for the classification of β -lactamases and our measured values

See the text for substrate profile details. Abbreviation: N.D., not determined.

Enzyme type	Substrate profile					
	According to Richmond & Sykes (1973)					
	Benzylpenicillin*	Ampicillin	Carbenicillin	Cloxacillin	Cephaloridine	Cephalexin
Ia	100	0	0	N.D.	8000	620
Ib	100	0	0	N.D.	350	80
Ic	100	150	N.D.	N.D.	2000	N.D.
Id	100	10	0	0	600	80
II	100	160–180	45	0	< 20	0
III	100	180	10	0	140	< 10
IV	100	120–170	10–50	< 10–20	50–150	0–< 10
V	100	100–950	60–80	0–200	20–120	< 10

Source of enzyme	According to our data. Basis: k_{cat} .					
<i>Enterobacter cloacae</i>	100	5	1×10^{-2}	2×10^{-2}	5000	500
<i>Citrobacter freundii</i>	100	20	0.5×10^{-2}	2×10^{-2}	2000	500
<i>Escherichia coli</i>	100	10	1×10^{-2}	1×10^{-2}	300	80
<i>Serratia marcescens</i>	100	3	N.D.	N.D.	1500	105
<i>Pseudomonas aeruginosa</i>	100	5	0.5×10^{-2}	1×10^{-2}	150	30

Source of enzyme	According to our data. Basis: k_{cat}/K_m					
<i>Enterobacter cloacae</i>	100	9	1	15	50	30
<i>Citrobacter freundii</i>	100	40	10	15	30	16
<i>Escherichia coli</i>	100	12	2	70	130	90
<i>Serratia marcescens</i>	100	100	N.D.	N.D.	10	6
<i>Pseudomonas aeruginosa</i>	100	20	0.5	2	10	7

* Reference compound: 100.

in class C enzymes. One can predict that the three-dimensional structures and catalytic or binding properties of the class C enzymes will be found to be very similar.

Does acyl-enzyme accumulate in all cases?

Knott-Hunziker *et al.* (1982) have presented very convincing evidence showing that, with benzylpenicillin and the *Pseudomonas ampC*-gene-product β -lactamase, deacylation was rate-limiting, resulting in acyl-enzyme accumulation. The generally low K_m values observed with most substrates indicated that the same rate-limiting step might prevail in most cases. With imipenem and aztreonam, accumulation of an intermediate, inactive with respect to the reporter substrate, could be directly visualized. However, in some cases (nitrocefin and the *Escherichia coli* K12 enzyme, cephaloridine and the *Escherichia coli* and *Serratia marcescens* enzymes, and cefazolin with all the enzymes) the K_m values were much larger. That might be due to strongly increased K values, but it is not impossible that, in those few cases, k_3 would not be completely rate-limiting, and the K_m value would more closely reflect that of K .

Correlation with the classification system of Richmond & Sykes (1973)

On the basis of our data, and in agreement with the previously proposed classification, our enzymes appeared

to belong to classes 1a, 1b and 1d of Richmond & Sykes (1973) Table 8 compares the reference values of those authors with those obtained in our study. We have computed the substrate profile according to both k_{cat} and k_{cat}/K_m . The enzymes studied here clearly belonged to class I if k_{cat} was chosen as the reference parameter. With k_{cat}/K_m such a classification was no longer justified. This was probably due to the fact that most of the early results reported 'activities' usually measured at concentrations above 100 μ M. Since most K_m values were below 100 μ M, the published 'activities' thus supplied rather good estimations of the relative values of k_{cat} . The transient inactivation that we observed with cloxacillin and carbenicillin had been interpreted as a 'powerful competitive inhibition' in earlier reports and corresponded in fact to a case of substrate competition where the steady state was not immediately reached with the 'inhibitory' substrate.

The distribution of the various enzymes in sub-classes 1a, 1b or 1d probably has a much more limited meaning. Indeed, none of the enzymes seems to exactly fit one of the sub-classes. For instance the *Enterobacter cloacae* and *Escherichia coli* K12 β -lactamases would nicely correspond to sub-classes 1a and 1b respectively if they did not hydrolyse ampicillin. This observation stresses again the need for an accurate measurement of the kinetic parameters. Indeed, and mainly in the earlier

literature, activities corresponding to less than 1% of that observed with the best substrate were often reported as 'zero'. As seen in Table 8, the k_{cat} of the *Enterobacter cloacae* β -lactamase with ampicillin is only 0.1% of that observed with cephaloridine, but it is, however, not negligible (0.6 s^{-1}). In conclusion, it seems that all members of Richmond & Sykes (1973) class I display some activity against ampicillin. It is, however, not very useful to try to distinguish sub-classes, since one can guess that the careful study of other enzymes will supply a possibly continuous spectrum of intermediate values. With such a situation, one might end up with one enzyme per sub-class! However, enzymes belonging to sub-classes 1a, 1b and 1d appear to share the following characteristics. With benzylpenicillin and k_{cat} as the reference substrate and parameter respectively: (1) lower k_{cat} with ampicillin (3–20%); (2) higher k_{cat} with cephaloridine (150–5000%); (3) similar k_{cat} with cephalixin (30–500%); (4) very low activities with carbenicillin and cloxacillin (0.5×10^{-2} – 2×10^{-2} %), with formation of a rather stable acyl-enzyme, resulting in (a) extremely low K_m values and (b) apparent competitive inhibition or transient inactivation when a reporter substrate is used; (5) slow inactivation by β -iodopenicillanate (100 – $400 \text{ M}^{-1} \cdot \text{s}^{-1}$) (De Meester *et al.*, 1986).

Above, in parentheses, are given the lower and upper values that we have observed, but they do not constitute absolute limits. Moreover, all those enzymes are recognized members of class C in the classification based on the primary structure. It will be interesting to see whether enzymes of sub-class 1c (mainly *Proteus vulgaris* enzymes) also share this latter characteristic. We believe that the study presented here will help to clarify the situation concerning the 'classification' of chromosome-coded 'cephalosporinases'.

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