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The diversity of the catalytic properties of class A β-lactamases

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The catalytic properties of four class A β-lactamases were studied with 24 different substrates. They exhibit a wide range of variation. Similarly, the amino acid sequences are also quite different. However, no relationships were found between the sequence similarities and the substrate profiles. Lags and bursts were observed with various compounds containing a large sterically hindered side chain. As a group, the enzymes could be distinguished from the class C β-lactamases on the basis of the $k_{cat.}$ values for several substrates, particularly oxacillin, cloxacillin and carbenicillin. Surprisingly, that distinction was impossible with the $k_{cat.}/K_m$ values, which represent the rates of acylation of the active-site serine residue by the β-lactam. For several cephalosporin substrates (e.g. cefuroxime and cefotaxime) class A enzymes consistently exhibited higher $k_{cat.}$ values than class C enzymes, thus belying the usual distinction between 'penicillinases' and 'cephalosporinases'. The problem of the repartition of class A β-lactamases into sub-classes is discussed.

INTRODUCTION

A large number of β-lactamases (EC 3.5.2.6) have already been described, and every year new members of that family are discovered. It is thus not surprising that various attempts have been performed to group those enzymes according to their structural or catalytic properties. Ambler (1980) has proposed a classification that relies on the primary structures of the proteins. On that basis four classes have now been identified. Classes A, C and D contain serine enzymes, and seem to represent the vast majority of β-lactamases, including those that pose the most threatening clinical problems. Class B contains a small number of Zn^{2+} enzymes, among which two closely related proteins produced by bacilli have been completely sequenced (Kato *et al.*, 1985; Hussain *et al.*, 1985).

Class C enzymes are chromosome-encoded and synthesized by Gram-negative bacteria, mainly Enterobacteriaceae and closely related species. The known sequences are highly conserved (Galleni *et al.*, 1988*a*), and surveys of the catalytic properties indicate very similar substrate profiles (Galleni & Frère, 1988; Galleni *et al.*, 1988*b*).

Although clearly homologous, the sequences of class A β-lactamases exhibit a much larger degree of variability. Alignments demonstrate that only a small number of residues are conserved throughout the dozen known primary structures.

Class A and C enzymes have the reputation to be 'penicillinases' and 'cephalosporinases' respectively, but the kinetic properties of class A β-lactamases also appear to be very variable. Rigorous comparisons are often difficult to perform since the kinetic parameters were not determined under identical conditions, the same substrates were not studied and purified preparations were not always utilized.

We have selected four class A enzymes of known primary structures produced by *Bacillus licheniformis*, *Actinomadura* R39, *Streptomyces cacaoi* and *Streptomyces albus* G. We have studied their catalytic properties on a representative sample of penicillins and cephalosporins. This could help to establish structure–activity relationships in class A and be a complement to the numerous site-directed mutagenesis experiments that have been initiated in various laboratories (Sigal *et al.*, 1984; Schultz & Richards, 1986; Madgwick & Waley, 1987).

Data about the three-dimensional structures of the *B. licheniformis* and the *Strep. albus* G enzymes are available (Kelly *et al.*, 1986; Dideberg *et al.*, 1987). The first of these is considered as the archetype of class A enzymes. Both its sequence and properties are very similar to those of β-lactamase I of *Bacillus cereus*, another well-studied enzyme. The sequence of the *Strep. albus* G enzyme is very different from that of the *B. licheniformis* enzyme, and, surprisingly, from that of the *Strep. cacaoi* enzyme, which is produced by a closely related organism (Dehottay *et al.*, 1987; Lenzini *et al.*, 1988). The gene coding for the *Actinomadura* R39 β-lactamase has recently been cloned (Piron-Fraipont *et al.*, 1989) and sequenced (Houba *et al.*, 1989). The deduced amino acid sequence exhibited 50% identities with that of the *B. licheniformis* enzyme.

Our comparison thus involves four class A β-lactamases of various degrees of relatedness.

MATERIALS AND METHODS

Determination of protein and enzyme activity

Routinely, protein concentrations were estimated by measuring the A_{280} of the solutions. More accurate determinations were performed by total hydrolysis with 6 M-HCl followed by reaction of the free amino groups

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with dinitrofluorobenzene as described in Duez *et al.* (1978).

The β-lactamase activity was determined by measuring the hydrolysis of nitrocefin. One unit represents the amount of enzyme that hydrolyses 1 μmol of nitrocefin/min, at maximal velocity, at pH 7 and 30 °C.

Enzymes

The β-lactamases were purified as follows.

***Enterobacter cloacae* P99 and 908R β-lactamases.** The Sephadex G-50 column in the procedure described by Ross (1975) was replaced by one of Sephadex G-100. The affinity-chromatography method of Cartwright & Waley (1984) was also used.

***Bacillus licheniformis* 749/C β-lactamases.** The procedure rested on that described by Thatcher (1975), but was extensively modified. The bacteria were grown overnight in a 500-litre tank. The conditions were those described by Frère *et al.* (1974) for *Actinomadura* R39. The medium contained, per litre: 4 g of peptone IBF, 4 g of casein hydrolysate, 0.25 g of $MgSO_4$, 0.8 g of K_2HPO_4, 0.6 g of KH_2PO_4, 1 mg of $FeSO_4,7H_2O$, 1 mg of $ZnSO_4,7H_2O$, 0.1 mg of $MnSO_4,4H_2O$, 0.01 mg of $CuSO_4,5H_2O$ and 4 μg of $K_2Cr_2O_7$. After centrifugation of the cells, 5 kg of Amberlite CG50 was added to the supernatant and the pH was adjusted to 4.5. After filtration, the ion-exchanger was suspended in 10 mM-ammonium acetate and the pH was adjusted to 8.5 with conc. NH_3. The supernatant was concentrated to 2 litres with the help of a Millipore Pellicon Cassette device and dialysed against water. The enzyme was adsorbed on a 200 ml column of CM-cellulose previously equilibrated at pH 4.5 in 10 mM-ammonium acetate. The column was then washed with 1 litre of 10 mM-ammonium acetate buffer, pH 8.5, and subsequently eluted with a linear gradient of 10–100 mM-ammonium acetate buffer, pH 8.5, over 1.5 litres. The active fractions were pooled, concentrated and divided into two samples, which were separately filtered through a 1-litre Sephadex G-100 column in 10 mM-Tris/HCl buffer, pH 7.2. A final purification step was performed on a 100 ml DEAE-Sepharose column equilibrated in 10 mM-Tris/HCl buffer, pH 7.2. The enzyme was eluted by applying a linear gradient of 0–200 mM-NaCl in 10 mM-Tris/HCl buffer, pH 7.2, over 500 ml. About 1 g of pure enzyme was thus obtained and the yield was 30%. The final preparation was dialysed against water and conserved at −20 °C, in solution or freeze-dried.

***Actinomadura* R39 β-lactamase.** The enzyme was purified as described by Piron-Fraipont *et al.* (1989).

***Streptomyces cacaoi* β-lactamase.** The enzyme was produced by *Strep. albus* G strain R2 (Chater & Wilde, 1980) harbouring plasmid pDML51 containing the gene coding for the *Strep. cacaoi* β-lactamase (Lenzini *et al.*, 1987). The medium contained, per litre: 3 g of yeast extract (Difco 0127–01), 5 g of bactopeptone (Difco 0118–01–8), 3 g of malt extract (Difco 0186–01–5), 10 g of glucose, 340 g of sucrose and 2 g of $CaCO_3$. Fifteen 1-litre conical flasks each containing 500 ml of medium were stirred at 28 °C during 10 days until a maximum enzyme concentration of 13 mg/l was obtained. The cells were eliminated by centrifugation and the enzyme was

Table 1. Purification of the extracellular β-lactamases from *Strep. cacaoi* and *Strep. albus* G

For experimental details see the Materials and methods section. The activities of both enzymes were measured with nitrocefin as substrate.

	Strep. cacaoi β-lactamase					*Strep. albus* G β-lactamase				
Purification step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification factor (fold)	Yield (%)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification factor (fold)	Yield (%)
Culture supernatant	261200	84000	3.1	–	100	286700	14400	20	–	100
DEAE-cellulose chromatography	146700	3600	41	13	56	236100	1310	180	9	82
Sephadex G-75 chromatography	117400	740	159	51	45	207100	240	863	44	72
Q-Sepharose fast-flow chromatography, pH 8	76100	130	586	189	29	148400	50	2970	149	52
Q-Sepharose fast-flow chromatography, pH 7	62800	34	1870	600	24	–	–	–	–	–
Chromatofocusing on MonoP	42200	16.9*	2500	800	16	101200	19.8*	5100	255	35

* Accurately determined by amino acid titration (see the Materials and methods section).

adsorbed on 400 g of DEAE-cellulose previously equilibrated in 10 mM-Tris/HCl buffer, pH 8. The DEAE-cellulose was used to prepare a column (7 cm diameter) that was washed with the same buffer containing 5% (v/v) glycerol and 5% (v/v) ethylene glycol (TGE buffer). The enzyme was eluted with a linear gradient of 0–0.25 M-NaCl in TGE buffer over 1200 ml. The active fractions were pooled and concentrated by ultrafiltration. The sample was filtered through a 1.5-litre Sephadex G-75 column in the same TGE buffer. After concentration, the enzyme was adsorbed on a 2.6 cm × 11 cm Q-Sepharose fast-flow column included in a Pharmacia f.p.l.c. system. Elution was performed with a linear gradient of 0–0.3 M-NaCl in a 20 mM TGE buffer over 900 ml. This step separated the *Strep. cacaoi* β-lactamase from the small amount (10%) of β-lactamase characteristic of the host strain. This chromatography step was repeated under the same conditions except that the pH of the buffer was adjusted to 7.0. Finally, a pure preparation was obtained by chromatofocusing on a MonoP HR5/20 column. The pH gradient went from 5.7 to 4.0. Buffer A was 25 mM-*N*-methylpiperazine/HCl buffer, pH 5.7, and buffer B was a 10-fold dilution of Polybuffer 74 (Pharmacia) adjusted to pH 4 with HCl and containing 5% (v/v) glycerol and 5% (v/v) ethylene glycol. In addition to inactive u.v.-absorbing material, this step separated four active peaks, three of which exhibited the same specific activity and behaved as homogeneous material upon SDS/polyacrylamide gel electrophoresis. This heterogeneity problem is not discussed further in the present paper. Table 1 summarizes the purification procedure. The final preparation was stored at 4 °C in 50 mM-sodium phosphate buffer, pH 7, containing 5% (v/v) glycerol and 5% (v/v) ethylene glycol.

***Streptomyces albus* G β-lactamase.** The enzyme was produced by *Strep. albus* G strain R2 harbouring plasmid pDML6. The culture medium was the same as that used above for production of the *Strep. cacaoi* β-lactamase. After 10 days at 28 °C, a total production of 60 mg was obtained in four conical flasks each containing 300 ml of medium. The purification procedure was the same as that described above except that the second chromatography on the Q-Sepharose fast-flow column at pH 7.0 was not necessary. As already described (Dehottay *et al.*, 1987), chromatofocusing also yielded several peaks of similar specific activity. The purification procedure is summarized in Table 1. The final preparation was conserved in the same buffer as for the *Strep. cacaoi* enzyme, but at −20 °C.

β-Lactam compounds

Benzylpenicillin was from Rhône–Poulenc (Paris, France), ampicillin and oxacillin were from Bristol Benelux (Brussels, Belgium), 6-aminopenicillanic acid, carbenicillin, cloxacillin, methicillin and ticarcillin were from Beecham Research Laboratories (Brentford, Middx., U.K.), cefotaxime was from Hoechst–Roussel (Romainville, France), cefamandole, cefazolin, cephalexin, cephaloglycin, cephaloridine, cephalosporin C and cephalothin were from Eli Lilly and Co. (Indianapolis, IN, U.S.A.), cephacetrile was from CIBA–GEIGY (Basel, Switzerland), ceftazidime and cefuroxime were from Glaxo Group Research (Greenford, Middx., U.K.) and 7-aminodeacetoxycephalosporanic acid was from Gist–Brocades (Delft, The Netherlands). All those compounds were kindly given by the respective companies. Penicillin V and penicillanic acid were gifts from Professor H. Vanderhaeghe and Professor P. Claes (Katholieke Universiteit, Leuven, Belgium). Nitrocefin was purchased from Oxoid (Basingstoke, Hants., U.K.) and 7-aminocephalosporanic acid from Janssen Pharmaceutica (Beerse, Belgium). The structures of the various compounds are shown in Fig. 1.

Determination of the kinetic parameters

Usually, a complete time course of the hydrolysis of the substrate was recorded at 482 nm for nitrocefin, at 260 nm for other cephalosporins, at 260 nm for oxacillin, cloxacillin and methicillin and at 235 nm for other penicillins. The values of $k_{cat.}$ and K_m were derived as described by De Meester *et al.* (1987). When the value of K_m was high, initial rates were determined and analysed according to the Hanes equation. In some cases K_m was so high that only the $k_{cat.}/K_m$ ratio was determined. When the K_m value was below 10 μM it was measured as a K_i with 100 μM-nitrocefin as substrate. In most of those latter cases $k_{cat.}/K_m$ for the substrate being tested was less than $0.2\,k_{cat.}/K_m$ for the reporter substrate. When that condition was not fulfilled, initial rates were measured so that less than 10% of both tested and reporter substrate were hydrolysed. The interactions between penicillanate and the *Strep. cacaoi* enzyme, between 7-aminocephalosporanic acid and the *B. licheniformis* enzyme and between ticarcillin or ceftazidime and the *Ent. cloacae* P99 enzyme were studied by using the reporter substrate method (De Meester *et al.*, 1987; Galleni & Frère, 1988). Further details are given in the Results section. All incubations were performed at 30 °C in 50 mM-sodium phosphate buffer, pH 7.0 (containing 5% glycerol and 5% ethylene glycol for the *Strep. albus* G and *Strep. cacaoi* β-lactamases). Dilutions of the enzymes below a concentration of 0.1 mg/ml were performed with buffer solutions containing 0.1 mg of bovine serum albumin/ml. The Beckman DU8 spectrophotometer was linked to an Apple II microcomputer (De Meester *et al.*, 1987). Standard deviations were computed on the basis of the results of five to ten experiments performed at various substrate and enzyme concentrations.

Thermal stability of β-lactamases

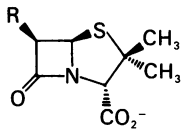
The enzymes were incubated at various temperatures in 50 mM-sodium phosphate buffer, pH 7.0, containing 0.1 mg of bovine serum albumin/ml. The buffer used in the experiments with the *Strep. albus* G and *Strep. cacaoi* β-lactamases also contained 5% glycerol and 5% ethylene glycol. The final enzyme concentrations were about 1 μg/ml. Samples were withdrawn after various periods of time and the residual activity was determined with nitrocefin as substrate at 30 °C.

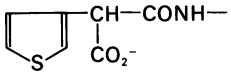
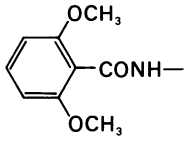
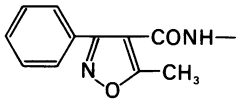
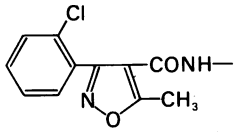
RESULTS

To obtain a wide overview of the catalytic profile of the various enzymes, compounds were selected on the basis of their structural characteristics and of their previously determined sensitivity to the various classes of β-lactamases.

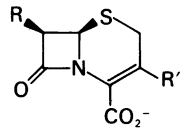
In the penicillin family, penicillanic acid and 6-aminopenicillanic acid have no acylamido side chain on C-6. Benzylpenicillin is usually used as the reference com-

Penicillins (penams)



R—	Name
H—	Penicillanic acid
NH_2—	6-Aminopenicillanic acid
C_6H_5—CH_2—CONH—	Benzylpenicillin
C_6H_5—CH(NH$_2$)—CONH—	Ampicillin
C_6H_5—CH(CO_2^-)—CONH—	Carbenicillin
C_6H_5—O—CH_2—CONH—	Penicillin V (phenoxymethylpenicillin)
	Ticarcillin
	Methicillin
	Oxacillin
	Cloxacillin

Cephalosporins (Δ^3-cephems)



R—	R′—	Name
NH_2—	CH_3—	7-Aminodeacetoxycephalosporanic acid
NH_2—	CH_3—CO—O—CH_2—	7-Aminocephalosporanic acid
C_6H_5—CH(NH_2)—CONH—	CH_3—	Cephalexin
C_6H_5—CH(NH_2)—CONH—	CH_3—CO—O—CH_2—	Cephaloglycin
N≡C—CH_2—CONH—	CH_3—CO—O—CH_2—	Cephacetrile
^-O_2C—CH(^+H_3N)—[CH_2]$_3$—CONH—	CH_3—CO—O—CH_2—	Cephalosporin C

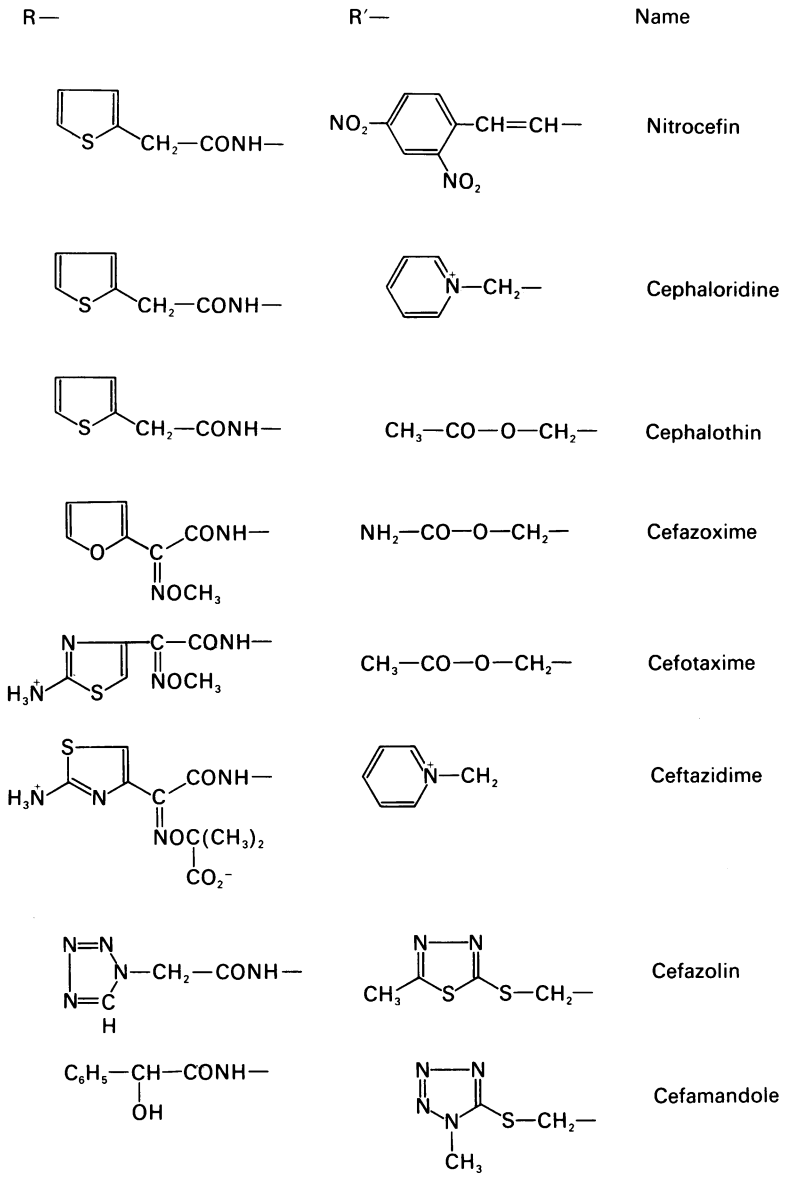


Fig. 1. Structures of the substrate molecules studied

Table 2. Kinetic parameters of β-lactamases for penicillanate and 6-aminopenicillanate

β-Lactamase	Penicillanate			6-Aminopenicillanate		
	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)
Actinomadura R39	63±7	37±4	590±50	57±5	450±25	8000±450
Strep. albus G	3300±300	200±15	61±3	200±10	720±40	3700±130
Strep. cacaoi	*	*	*	300±15	120±4	400±15
B. licheniformis	490±30	21±4	50±10	9±0.3†	57±2†	6000±400†
Ent. cloacae P99	N.D.‡	N.D.‡	N.D.‡	Complex branched pathways§		

* Inactivation by acyl-enzyme accumulation. See the text.

† Lag. The K_m was obtained by substrate competition by measuring the steady-state rate of nitrocefin hydrolysis. The $k_{cat.}$ value was measured directly at 1 mM after establishment of the steady state.

‡ Not determined.

§ D. Monnaie, M. Galleni & J.-M. Frère (unpublished work).

pound, and phenoxymethylpenicillin, ampicillin, carbenicillin and ticarcillin are usually considered as fair to good substrates of most ‘penicillinases’. On the other hand, cloxacillin, oxacillin and methicillin with their bulky side chains generally appear to be poorer substrates and can also be responsible for substrate-induced inactivation (Citri *et al.*, 1976; Kiener & Waley, 1977; Kiener *et al.*, 1980). Among the many available cephalosporins, we also selected 7-aminocephalosporanic acid and its deacetoxy equivalent, both devoid of acyl substituent on the amino group on C-7. Various compounds were considered as good substrates of class C enzymes: nitrocefin, cephaloridine, cephalothin, cephacetrile, cephaloglycin, cephalosporin C and cefazolin. Cephalexin was also included because it has the same C-7 side chain as cephaloglycin, but, similarly to 7-aminodeacetoxycephalosporanic acid, only a methyl group on C-3. Cefuroxime, cefotaxime and ceftazidime have an oxyimino group on the C-7 side chain and are considered to be ‘β-lactamase-resistant’. The chosen compounds represented the three generations of cephalosporins, with cefamandole and cefuroxime for the second and cefotaxime and ceftazidime for the third generations respectively. The results are presented in Tables 2–9. For penicillins and the P99 β-lactamase, some values of the parameters were those measured by Galleni & Frère (1988) at pH 8.2. However, the kinetic parameters of class C β-lactamases did not vary by more than 3-fold (Bicknell *et al.*, 1983; Joris *et al.*, 1986) between pH 8.2 and 7. With cephalexin it appeared that the primary hydrolysis product was not stable under our experimental conditions and decayed with an apparent first-order rate constant of $2\times10^{-3}\ s^{-1}$, resulting in an additional decrease of A_{260}. In consequence, the kinetic parameters were deduced from the measurement of initial rates, obtained over very short periods of 2–3 min.

Penicillanate was not significantly hydrolysed by the *Strep. cacaoi* enzyme ($k_{cat.} < 4\times10^{-4}\ s^{-1}$), but it behaved as an inactivator. The pseudo-first-order rate constant for the inactivation was measured with various penicillanate concentrations, and values of $0.1\pm0.01\ s^{-1}$ and 5.0 ± 0.05 mM were found for k_{+2} and K' respectively on the basis of the simple pathway (Fig. 2):

$$E + C \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EC \xrightarrow{k_{+2}} EC^*$$

The same phenomenon was observed in the interaction between the P99 β-lactamase and ticarcillin. No hydrolysis was observed ($k_{cat.} < 10^{-4}\ s^{-1}$) and the value of $85000\pm10000\ M^{-1}\cdot s^{-1}$ was measured for k_2/K'.

Lags or bursts were observed for various interactions. In some cases the same phenomenon was suspected but could not be identified with certainty. Under our experimental conditions the mixing dead-time was 5–10 s, and any lag or burst that would be completed within a shorter time could not be detected. With methicillin such a non-classical behaviour had already been reported for the *B. cereus* serine β-lactamase and various class C enzymes (Citri *et al.*, 1976; Galleni & Frère, 1988).

Surprisingly, instead of the expected behaviour, i.e. substrate-induced inactivation, a lag, characteristic of substrate-induced activation, was observed upon hydrolysis of methicillin by the four class A β-lactamases studied in the present work (Fig. 3). For the *B. licheniformis* β-lactamase and after reaching the steady state, a

Table 3. Kinetic parameters of β-lactamases for benzylpenicillin, ampicillin, phenoxymethylpenicillin and carbenicillin

β-Lactamase	Benzylpenicillin			Ampicillin			Phenoxymethylpenicillin			Carbenicillin		
	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3}\times k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$)	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3}\times k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$)	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3}\times k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$)	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3}\times k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$)
Actinomadura R39	50±5	370±30	7500±1000	120±20	1900±200	16000±1500	85±10	830±35	9900±900	250±20	220±15	870±25
Strep. albus G	1000±60	2800±200	2800±100	650±50	3900±200	6100±400	1000±200	2800±450	2900±300	>10000	>1000	100±15*
Strep. cacaoi	96±6	1050±130	11000±1000	52±4	310±25	5700±600	50±6	770±100	15000±1500	100±10	920±50	8800±800
B. licheniformis	76±5	2200±100	29000±2000	143±8	1500±100	11000±600	39±3	1000±70	26000±2000	51±2	400±10	8100±200
Ent. cloacae P99†	0.6±0.1	14±1.5	23000±5000	0.4±0.05	0.7±0.05	1800±400	4±0.2	8±0.4	2000±100	≃0.01	≃2×10^{-3}	260±30

* Determined by using a first-order time course at [S] ≪ K_m. The time course remained first-order up to the concentration given in the K_m column.

† Data obtained by Galleni & Frère (1988) at pH 8.2, except for phenoxymethylpenicillin, for which data were obtained at pH 7. In this case the K_m was obtained by substrate competition and the $k_{cat.}$ value was determined by measuring the initial rate at 2 mM.

Table 4. Kinetic parameters of β-lactamases for oxacillin, cloxacillin and ticarcillin

β-Lactamase	Oxacillin			Cloxacillin			Ticarcillin		
	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)
Actinomadura R39	160±15*	940±50*	5900±600*	25±3*	150±10*	6200±750*	240±40	190±25	800±65
Strep. albus G	430±50	270±40	630±60	450±60	190±10	440±40	> 500†	> 60†	125±15†
Strep. cacaoi	700±80	2000±150	2800±120	220±25‡	630±50‡	3000±350‡	130±20	1200±120	9500±800
B. licheniformis	8±0.2§	10±0.5§	1200±100§	11±0.5§	8.5±0.3§	775±25§	46±2	220±10	4700±400
Ent. cloacae P99	4×10^{-4}‖	5×10^{-3}‖	7000±500‖	4×10^{-4}‖	5×10^{-3}‖	11000±700‖	¶	¶	¶

* Lag. Values were obtained by neglecting the non-linear part of the curve.

† Determined by using a first-order time course at [S] ≪ K_m. The time course remained first-order up to the concentration given in the K_m column.

‡ Possible lag. Any lag shorter than 10 s remains difficult to see with the method utilized in this study.

§ See the text. K_m values were determined as K_i values by substrate competition. $k_{cat.}$ values were determined by measuring the initial rate at [S] ≫ K_m.

‖ Data obtained by Galleni & Frère (1988) at pH 8.2.

¶ Inactivation by acyl-enzyme accumulation. See the text.

Table 5. Kinetic parameters for β-lactamases for nitrocefin, cephaloridine and cephalothin

β-Lactamase	Nitrocefin			Cephaloridine			Cephalothin		
	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)
Actinomadura R39	70±5	600±65	8500±400	38±3	440±45	11600±700	54±3	200±25	3700±300
Strep. albus G	1100±140	2500±300	2400±110	320±25	200±10	620±50	720±40	260±20	370±20
Strep. cacaoi	1300±120	1050±100	800±70	1050±200	260±40	250±17	960±50	11±0.5	11.5±0.6
B. licheniformis	38±5	470±20	13000±400	135±6	630±30	5000±500	20±1	48±2	2500±30
Ent. cloacae P99	75±5	800±60	10500±1000	90±5	500±20	5600±300	29±4	160±8	5500±200

K_m of 1.3±0.03 μM was measured by substrate competition and a $k_{cat.}$ of 11±0.2 s^{-1} at substrate saturation (1 mM). With the Actinomycetes enzymes it was estimated that the $k_{cat.}$ value was larger than 10 s^{-1}, which was always much larger than what was observed with the class C enzymes (0.01–0.1 s^{-1} after completion of the 'burst').

Oxacillin and cloxacillin also induced a lag with the three Actinomycetes enzymes. After the progressive increase in the hydrolysis rate was completed, remarkably high $k_{cat.}$ values were measured (Table 4). With the *B. licheniformis* enzymes the two K_m values were obtained by substrate competition and the $k_{cat.}$ values by measuring the initial rate at a 1 mM concentration of substrate (i.e. approx. 100 K_m). However, under those conditions a slight progressive slowdown of the reaction was observed when a complete hydrolysis time course was recorded, but that phenomenon was not further investigated.

Thermal stability

Surprisingly, and as observed by Schultz *et al.* (1987), the half-lives of the enzymes decreased when their concentrations increased. For instance, with the *Strep. albus* G β-lactamase at 45 °C the half-lives were respectively 30 min at 1 mg/ml and 53 min at 1 μg/ml. With the *Escherichia coli* K12 chromosome-encoded class C β-lactamase at 53 °C the half-life was 30 min at 0.4 mg/ml and 41 min at 4 μg/ml. In both cases the half-lives of the diluted samples did not depend upon the presence of bovine serum albumin (0.1 mg/ml) in the dilution buffer. The surprising concentration effect was thus more probably due to the β-lactamase concentration itself than to the total protein concentration.

Table 10 compares the stabilities of seven class A and five class C enzymes at various temperatures. The data for the *Citrobacter diversus*, *B. licheniformis* and *Esch. coli* RTEM class A β-lactamases were taken from previously published work (Amicosante *et al.*, 1988; De Meester *et al.*, 1987; Schultz *et al.*, 1987). Inactivation of the P99 β-lactamase did not obey first-order kinetics. Fig. 4 compares the inactivations of the P99 and 908R β-lactamases.

DISCUSSION

To complement our analysis, Table 11 summarizes the published values of the kinetic parameters for three other class A β-lactamases.

After a cursory examination of Tables 2–9 and 11, one might be tempted to conclude that little useful information can be obtained by comparing the kinetic parameters of the various class A enzymes. Indeed, each enzyme appears to exhibit a very individual behaviour. The available sequences were compared with the help of statistical methods (Table 12), which showed, as expected, a strong similarity between the *B. licheniformis* and *Actinomadura* R39 enzymes (55% identical residues, no insertions). Conversely, the RTEM-2 and *Staphylococcus*

Table 6. Kinetic parameters of β-lactamases for 7-aminodeacetoxycephalosporanic acid, 7-aminocephalosporanic acid and cephalexin

β-Lactamase	7-Aminodeacetoxycephalosporanic acid			7-Aminocephalosporanic acid			Cephalexin*		
	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)
Actinomadura R39	> 200†	> 0.06†	0.29 ± 0.02†	> 2000†	> 5†	2.6 ± 0.2†	1300 ± 100‡	285 ± 15‡	220 ± 20‡
Strep. albus G	> 200†	> 10^{-3}†	(6 ± 0.6) × 10^{-3}†	> 3000†	> 0.5†	0.17 ± 0.01†	7300 ± 400§	12 ± 1§	1.7 ± 0.1§
Strep. cacaoi	> 1000‖	< 5 × 10^{-3}‖	< 20 × 10^{-3}‖	> 200†	> 0.01†	0.06 ± 0.004†	> 2000†	> 2†	0.9 ± 0.1†
B. licheniformis	2200 ± 200¶	(2 ± 0.2) × 10^{-3}¶	(0.8 ± 0.07) × 10^{-3}¶	220 ± 20**	0.07 ± 0.004**	0.33 ± 0.03**	120 ± 5‡	5 ± 0.2‡	40 ± 5‡
Ent. cloacae P99	4700 ± 500¶	0.08 ± 0.02¶	(17 ± 3) × 10^{-3}¶	950 ± 30‡	0.2 ± 0.05‡	0.2 ± 0.1‡	95 ± 5‡	85 ± 5‡	875 ± 50‡

* All values were determined by measuring initial rates.
† Determined by using a first-order time course at [S] ≪ K_m. The time course remained first-order up to the concentration given in the K_m column.
‡ Values determined by using the Hanes plot.
§ Lag. Values obtained by neglecting the non-linear part of the curve and using Hanes' plot.
‖ 0.02 mg of *Strep. cacaoi* β-lactamase in 500 μl of 200 μM-7-aminodeacetoxycephalosporanic acid gave no detectable hydrolysis after 1 h at 30 °C. With nitrocefin as substrate, no inhibition was detected in the presence of 1 mM-7-aminodeacetoxycephalosporanic acid.
¶ K_m values were determined as K_i values by substrate competition. $k_{cat.}$ values were determined by measuring the initial rate at [S] = K_m (*B. licheniformis* enzyme) or [S] = $K_m/2$ (*Ent. cloacae* P99 enzyme).
** $k_{cat.}/K_m$ was determined as k_{+2}/K' by using the reporter substrate method. Only partial inactivation was observed. From the steady state the value of K_m could be measured. The $k_{cat.}$ value was determined by monitoring directly the 7-aminocephalosporanic acid hydrolysis and measuring the initial rate at [S] ≫ K_m.

Table 7. Kinetic parameters of β-lactamases for cephacetrile, cephaloglycin and cephalosporin C

β-Lactamase	Cephacetrile			Cephaloglycin			Cephalosporin C		
	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)
Actinomadura R39	190 ± 15	500 ± 25	2700 ± 80	940 ± 80	2500 ± 130	2700 ± 100	1200 ± 150	500 ± 50	420 ± 20
Strep. albus G	⩾ 6000	⩾ 450	76 ± 3	> 3000*	> 100*	32 ± 1*	4500 ± 1000†	170 ± 30†	40 ± 1†
Strep. cacaoi	3500 ± 400	21 ± 2	6 ± 0.15	> 3000*‡	> 10*‡	3.5 ± 0.3*‡	> 2000*	> 1*	0.5 ± 0.05*
B. licheniformis	10 ± 1	28 ± 2	2900 ± 400	39 ± 3	34 ± 0.8	900 ± 80	98 ± 5	14 ± 2	150 ± 20
B. cloacae P99	140 ± 10	143 ± 8	1000 ± 20	2 ± 0.2§	0.9 ± 0.08§	430 ± 35§	400 ± 20	1100 ± 100	2700 ± 200

* Determined by using a first-order time course at [S] ≪ K_m. The time course remained first-order up to the concentration given in the K_m column.
† Possible lag. Any lag shorter than 10 s remains difficult to see with the method utilized in this study.
‡ Lag. Values were obtained by neglecting the non-linear part of the curve.
§ K_m values were determined as K_i values by substrate competition. $k_{cat.}$ values were determined by measuring the initial rate at [S] ≫ K_m.

Table 8. Kinetic parameters of β-lactamases for cefuroxime, cefotaxamine and ceftazidime

β-Lactamase	Cefuroxime			Cefotaxime			Ceftazidime		
	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)
Actinomadura R39	170±20	110±10	640±70	680±70	280±40	400±40	> 1000*	> 13*	13±1*
Strep. albus G	> 1000*	> 25*	25±2*	> 1000*	> 1*	1±0.05*	> 1000†	< 10^{-3}†	< 10^{-3}†
Strep. cacaoi	> 1000*	> 2*	1.8±0.1*	> 1000*	> 0.5*	0.5±0.05*	> 1000*	> 3*	3.5±0.3*
B. licheniformis	93±5	16±1	170±30	205±5	6.7±0.2	33±8	1400±150	15±1	9±0.2
E. cloacae P99	0.01±0.001‡	0.06±0.01‡	6000±1000‡	0.033±0.008‡	0.035±0.006‡	1100±200‡	4±0.4§	0.012±0.003§	2.5±0.2§

* Determined by using a first-order time course at [S] ≪ K_m. The time course remained first-order up to the concentration given in the K_m column.
† 0.3 mg of *Strep. albus* G β-lactamase in 400 μl of 1 mM-ceftazidime gave no detectable hydrolysis after 5 min at 30 °C. With nitrocefin as substrate no inhibition was detected in the presence of 1 mM-ceftazidime.
‡ K_m values were determined as K_i values by substrate competition. $k_{cat.}$ values were determined by measuring the initial rate at [S] ≫ K_m.
§ $k_{cat.}/K_m$ was determined as k_{+2}/K' by using the reporter substrate method. Only partial inactivation was observed. From the steady state the value of K_m could be measured. The $k_{cat.}$ value was determined by monitoring directly the ceftazidime hydrolysis and measuring the initial rate at [S] ≫ K_m.

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} ES^* \xrightarrow{k_{+3}} E + P$$

Scheme 1. Interaction between a serine β-lactamase and substrate

ES* represents the acyl-enzyme, $K' = (k_{-1} + k_{+2})/k_{+1}$ and $k_{cat.}/K_m = k_{+2}/K'$.

aureus β-lactamases appear to be further relatives of the four enzymes studied here. Strikingly, these two enzymes are also very different from each other.

The 'central' position of the *B. licheniformis* β-lactamase is also remarkable, since it is consistently the protein that exhibits the highest score with any other one.

Fig. 5 presents an attempt to depict graphically the relationships between the four class A β-lactamases studied here.

There does not seem to be a clear relationship between those sequence similarities and the specificity profiles of the different enzymes: a pairwise comparison fails to detect any particular pattern in the values of $k_{cat.}$ and $k_{cat.}/K_m$.

However, a careful study of our data reveals several interesting details, deserving further discussion. The analysis rests on the model shown in Scheme 1, which is widely accepted for serine β-lactamases.

Variability in class A β-lactamases

The range of variation of the kinetic parameters for a given β-lactam is generally quite wide, but it is much wider for cephalosporins than for penicillins. Strikingly, the $k_{cat.}/K_m$ values for all tested cephalosporins span two or three orders of magnitude with only four enzymes! The behaviour of the same enzymes towards penicillins is much more homogeneous, and all the tested penicillins are good substrates, including cloxacillin and oxacillin. The only striking exception is the interaction between penicillanic acid and the *Strep. cacaoi* β-lactamase, in which the antibiotic behaves as a rather inefficient inactivator ($k_2/K' = 20\ M^{-1} \cdot s^{-1}$ and $k_3 \leqslant 10^{-4}\ s^{-1}$). The $k_{cat.}/K_m$ values for the hydrolysis of carbenicillin and ticarcillin by the *Strep. albus* G enzyme are also distinctively lower (about $100000\ M^{-1} \cdot s^{-1}$) than for the three other enzymes. If one includes the published values presented in Table 11, these general conclusions are not modified. The RTEM-2 enzyme exhibits higher values of $k_{cat.}/K_m$ with most penicillins, mainly because of its lower K_m values. Of the few available values for the *Staph. aureus* enzyme, two are strikingly lower than those reported for the enzymes studied here: the $k_{cat.}/K_m$ ratios for 6-aminopenicillanic acid and cloxacillin.

Comparison with class C β-lactamases

The inclusion of the *Ent. cloacae* P99 β-lactamase in the comparison yields the most surprising result of the analysis: the $k_{cat.}/K_m$ values for this enzyme nearly always fall within the range of variation of the same parameter for the class A enzymes. When it does not (with ampicillin, cephalothin, cephalexin, cephalosporin C, cefuroxime and cefotaxime) it is never distant from the nearest class A value by more than 10-fold. This result indicates that, if one considers the rate of acylation, there is no clear distinction between both classes of β-lactamases. With cephalosporins, that observation is certainly correlated to the wide range of variation of the

Table 9. Kinetic parameters of β-lactamases for cefazolin and cefamandole

β-Lactamase	Cefazolin			Cefamandole		
	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)
Actinomadura R39	70±2	260±15	3700±300	310±30	720±35	2300±200
Strep. albus G	2800±200*	1500±100*	560±20*	1600±200†	800±100†	500±30†
Strep. cacaoi	⩾3000	⩾100	33±3	1550±150‡	65±2‡	43±4‡
B. licheniformis	12±2	300±10	25000±2000	12±1	58±3	4800±80
Ent. cloacae P99	3200±200	1800±200	560±100	19±3	11±0.5	600±50

* Also determined by the Hanes plot.
† Lag. Values were obtained by neglecting the non-linear part of the curve.
‡ Possible lag. Any lag shorter than 10 s remains difficult to see with the method utilized in this study.

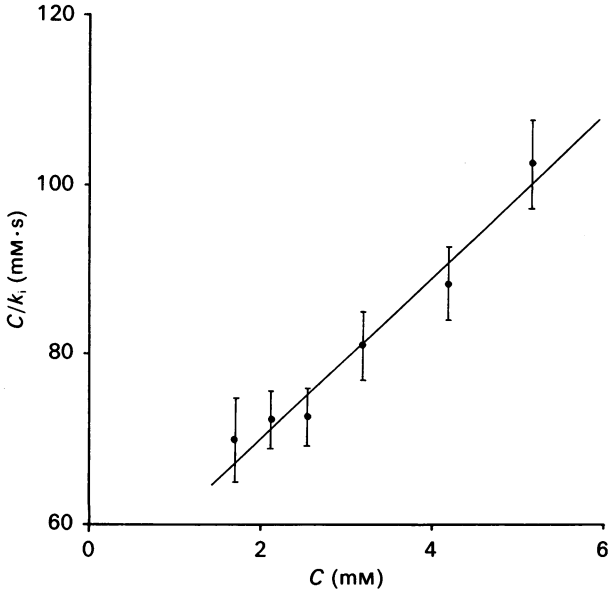


Fig. 2. Inactivation of the *Strep. cacaoi* β-lactamase by penicillanate

Plot of C/k_i versus C, where C is the concentration of penicillinate and k_i is the pseudo-first-order inactivation rate constant. Nitrocefin was the reporter substrate. To 450 μl of 100 μM-nitrocefin in 50 mM-sodium phosphate buffer, pH 7.0, containing various concentrations of penicillinate, 0.06–0.14 μg portions of enzyme were added. The value of k_i was computed by analysing the progressive decrease of the nitrocefin hydrolysis rate. $K' = (k_{-1}+k_{+2})/k_{+1}$.

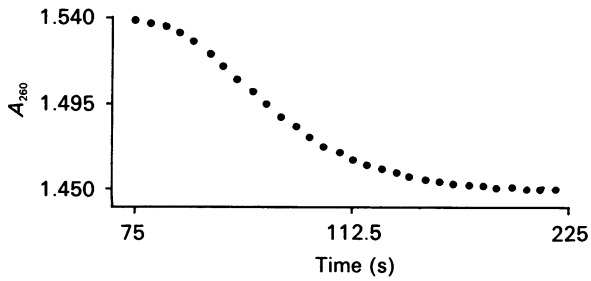


Fig. 3. Time course of the hydrolysis of methicillin by the *Strep. albus* G β-lactamase

To 450 μl of 1 mM-methicillin in 50 mM-sodium phosphate buffer, pH 7.0, 10 μg of enzyme was added. The value of A_{260} was monitored at two readings per s. Afterwards, the values of 15 successive readings were averaged, yielding one value per 7.5 s.

class A $k_{cat.}/K_m$ values. But this is not a sufficient explanation. Indeed, and although the class A $k_{cat.}$ values are also widely variable, the $k_{cat.}$ values of the class C enzymes (Galleni & Frère, 1988; Galleni *et al.*, 1988*b*) are consistently lower than those of class A for the following compounds: benzylpenicillin, ampicillin, oxacillin, carbenicillin, cefuroxime, cefotaxime and, if one excepts the *C. diversus* enzyme, cloxacillin, the difference being particularly large with oxacillin, cloxacillin and carbenicillin. This is also probably true for methicillin, but the comparison is more difficult for that compound since the lags observed did not allow us to measure the kinetic parameters accurately. It is particularly interesting to compare the behaviour of the *Strep. albus* G and P99 enzymes towards carbenicillin. Although the $k_{cat.}/K_m$ values are similar (100000 and 260000 $M^{-1} \cdot s^{-1}$ respectively), the enzymes exhibit respectively a very high ($> 1000\ s^{-1}$) and a very low ($2 \times 10^{-3}\ s^{-1}$) $k_{cat.}$ value. Accordingly, the respective K_m values are extremely high and extremely low. One can thus conclude that the major difference between class A and class C enzymes is at the level of the deacylation step. Poor substrates of class C enzymes always appear to form stable acyl-enzyme intermediates, a property similar to those of penicillin-sensitive enzymes. With the class A enzymes, low values of $k_{cat.}$ appear to be more exceptional and, at least with the three Actinomycetes enzymes, are often correlated to high K_m values. This indicates high K' values (in the millimolar range or higher) and suggests that one should probably not expect severely rate-limiting deacylations in those cases. The K_m values are further discussed in a following paragraph. At this point, however, it may be noted that the only compounds for which a distinctly slower acylation has been demonstrated for class C β-lactamases are the inactivators β-iodopenicillanate (De Meester *et al.*, 1986) and clavulanate (Reading & Farmer, 1981). However, a detailed analysis of the interaction between the latter compound and class C β-lactamases has not been performed.

Influence of the structures of the side chains

All the class A enzymes hydrolyse benzylpenicillin, penicillin V and ampicillin with a high efficiency, where the rates of both acylation and deacylation appear to be high. The amino group of ampicillin does not appear to

Table 10. Half-lives of various class A and class C β-lactamases at different temperatures

Data for the *B. licheniformis* enzyme were obtained from De Meester *et al.* (1987), those for the *C. diversus* enzyme from Amicosante *et al.* (1988), and those for the *Esch. coli* RTEM enzyme from Schultz *et al.* (1987). The error on the different values is usually lower than 10%.

		Half-life (min)						
β-Lactamase	Temperature...	40 °C	45 °C	50 °C	55 °C	60 °C	65 °C	70 °C
Class A								
Actinomadura R39		11	2					
Streptomyces albus G		⩾ 300	55	7				
Streptomyces cacaoi			53	15				
Bacillus licheniformis			> 60*	> 60*	> 60*	> 60*	45 (64 °C)	1.3
Klebsiella pneumoniae			50	17	2.3			
Citrobacter diversus		29	10	< 3				
Escherichia coli RTEM			> 1200*	500	13	1.5		
Class C								
Serratia marcescens					31	5		
Escherichia coli K12					10	2.5		
Enterobacter cloacae 908R					> 120*	29		
Pseudomonas aeruginosa					3			
Citrobacter freundii					14	< 2		

* Enzymes retained more than 90% of their initial activity after the indicated time had elapsed.

have a specific effect. Although the $k_{cat.}$ values are generally somewhat lower, carbenicillin and ticarcillin are also good substrates. The distinctly lower $k_{cat.}/K_m$ value of the *Strep. albus* G β-lactamase for these substrates is due to a high K_m, as discussed above. Thus in class A the presence of an amino or carboxylate group on the side chain does not appear to significantly modify the acylation and deacylation rates. In class C, by contrast, the amino group decreases the k_{+3} value by one and the carboxylate group by three or four orders of magnitude. A similar observation can be made with oxacillin, cloxacillin and methicillin, which have large, sterically hindered, side chains. With class C enzymes, these compounds exhibit large $k_{cat.}/K_m$ and very low $k_{cat.}$ values, which makes them transient inactivators. Conversely, they are fair or good substrates of the class A enzymes ($k_{cat.} \geqslant 8\ s^{-1}$) with the exception of the *C. diversus* β-lactamase and cloxacillin. The very low $k_{cat.}/K_m$ value observed with the *Staph. aureus* enzyme ($800\ M^{-1} \cdot s^{-1}$) is, in fact, due to an extremely high K_m value.

The removal of the acyl group on the C-6 or C-7 side chain of penicillins or cephalosporins respectively has a very different consequence. With penicillins, 6-aminopenicillanic acid remains a good substrate, with rather high acylation and deacylation rates. For the *Actinomadura* R39 β-lactamase 6-aminopenicillanic acid is an even better substrate than benzylpenicillin. This is in sharp contrast with the situation that prevails with most penicillin-binding proteins, for which 6-aminopenicillanic acid is a very poor inactivator. It may be noted, however, that with the *Staph. aureus* β-lactamases the $k_{cat.}/K_m$ value for 6-aminopenicillanic acid is only 0.1% of that observed with benzylpenicillin.

With cephalosporins, the situation is quite different. A comparison of the pairs ampicillin/6-aminopenicillanic acid, cephalexin/7-aminodeacetoxycephalosporanic acid and cephaloglycin/7-aminocephalosporanic acid shows that the removal of the C_6H_5–$CHNH_2$–CO side chain decreases the $k_{cat.}/K_m$ values for the cephalosporins in a dramatic way for all the enzymes, whereas for the penicillins a comparable effect is only observed with the *Staph. aureus* enzyme.

Class A β-lactamases: penicillinases or cephalosporinases?

Class A and class C enzymes are generally considered to be 'penicillinases' and 'cephalosporinases' respectively. If one compares the behaviour of class A enzymes

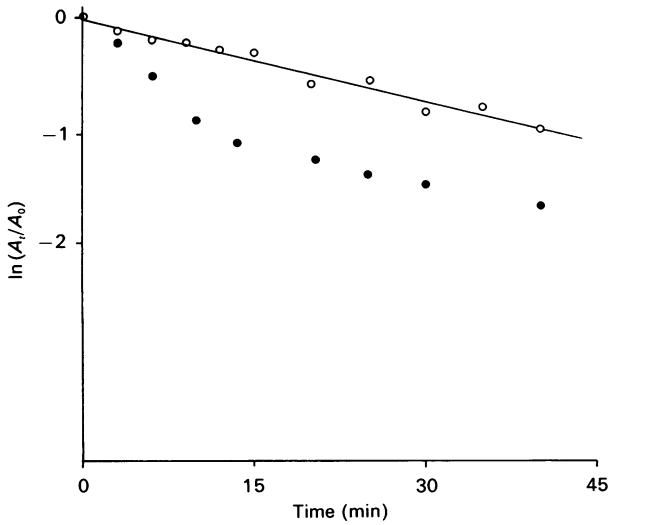


Fig. 4. Thermal inactivation of the P99 β-lactamase (●) and 908R β-lactamase (○) at 60 °C

A_0 and A_t represent the activities at times 0 and t respectively. The enzymes were incubated at a concentration of 3 μg/ml in 50 mM-sodium phosphate buffer, pH 7.0, containing 0.1 mg of bovine serum albumin/ml. After various periods of time samples were withdrawn and the residual activity was measured at 30 °C with 100 μM-nitrocefin as substrate.

Table 11. Published kinetic parameters of some other class A β-lactamases

Data for the RTEM-2 enzyme were obtained from Fisher *et al.* (1981) (for penicillanic acid) and Labia *et al.* (1979) (for all other substrates), those for the *C. diversus* enzyme from Amicosante *et al.* (1988), and those for the *Staph. aureus* enzyme from R. Virden (personal communication) (for nitrocefin) and Richmond (1975) (for all other substrates).

	RTEM-2 β-lactamase			*C. diversus* β-lactamase			*Staph. aureus* β-lactamase*		
Substrate	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)
Penicillanic acid		40							
6-Aminopenicillanic acid							70	40	600
Benzylpenicillin	15–20	2000	100000–140000	70	570	8300	5	400	80000
Penicillin V	10	1300	130000				7	440	60000
Ampicillin	22	1900	87000	22	120	5400	17†	750	50000
Carbenicillin	9	220	25000	100	60	600			
Ticarcillin	7	220	30000						
Oxacillin	5	90	20000	230	206	900			
Cloxacillin	10	26	2600	< 100	0.04	> 400	10000	8	0.8
Methicillin		7					10000	6	0.6
Nitrocefin	55	550	10000				1 (0 °C)	2.6 (0 °C)	2600 (0 °C)
Cephaloridine	2100	6300	3000	740	900	1250	500‡	40‡	80‡
Cephalothin	350	540	1500	50	63	1300			
Cephaloglycin	400	420	1000						
Cefazolin	680	680	1000	20	170	8300			
Cefamandole	670	1800	3000						
Cephalosporin C				130	41	300	100	5	50
Cefuroxime	1000	3	3	70	54	770			
Cefotaxime	3000	7	2.1						

* The values of Richmond (1975) were apparently computed on the basis of an incorrect absorption coefficient and should be divided by 2 (R. Virden, personal communication).
† A. L. Fink & R. Virden (personal communication) report a K_m value of 200 μM at 0 °C.
‡ These values should be considered with caution since progressive inactivation was detected by De Meester *et al.* (1986) and Carrey *et al.* (1984).

Table 12. Analysis of the sequence similarities between six class A β-lactamases

In addition to the four enzymes examined in our study, the RTEM-2 and *Staph. aureus* β-lactamases have been included in the analysis. In each column, the best and worst scores are underlined with continuous and discontinuous lines respectively. The comparisons were performed by using the Goad & Kanehisa (1982) procedures as done before by Joris *et al.* (1988) and were initiated at residue 31 in the Ambler (1980) numbering, where the active-site serine residue is in position 70. This type of analysis involves a penalty for the introduction of deletions or insertions. The lower the score, the higher the similarity. The proteins were also aligned pairwise and the numbers of identical amino acids determined. As expected, the *B. licheniformis* and *Actinomadura* R39 β-lactamases had the highest number of identical residues (55%), whereas only 32% and 33% of the *Staph. aureus* enzyme residues were identical with those of the *Strep. albus* G and RTEM-2 β-lactamases, respectively. All other comparisons yielded 37–48% of identical residues.

	RTEM-2	*Staph. aureus*	*Strep. albus* G	*Strep. cacaoi*	*Actinomadura* R39	*B. licheniformis*
RTEM-2		−370	−446	−428	−409	−491
Staph. aureus	−370		−332	−444	−462	−592
Strep. albus G	−446	−332		−470	−460	−512
Strep. cacaoi	−428	−444	−470		−531	−555
Actinomadura R39	−409	−462	−460	−531		−754
B. licheniformis	−491	−592	−512	−555	−754	

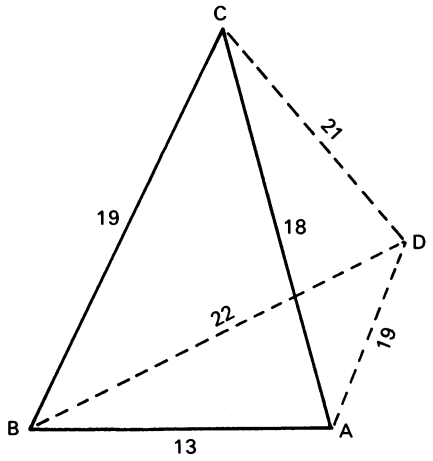


Fig. 5. Graphical representation of the distances between the four class A β-lactamases studied here

Key: A, *B. licheniformis* enzyme; B, *Actinomadura* R39 enzyme; C, *Strep. cacaoi* enzyme; D, *Strep. albus* G enzyme. The distances have been arbitrarily computed as $10000/|y|$, where y is the value found in the pairwise comparisons of Table 12. In this representation the *Staph. aureus* enzyme would be on the reader's side of plane ABC, thus far away from the *Strep. albus* G enzyme (30), and the RTEM-2 β-lactamase would be on the opposite side of plane BCD, far away from the *Staph. aureus* enzyme (27) and somewhat closer to the *B. licheniformis* enzyme (20) than to the three other ones (22, 23 and 24).

towards ampicillin and cephaloglycin or cephalexin, that assumption might appear justified: the two cephalosporins are usually poorer substrates, although the differences with the *Actinomadura* R39 β-lactamase are not extremely large. However, cephaloridine and nitrocefin are good substrates of all the enzymes, and again the individual behaviour of the class A β-lactamases is striking. The $k_{cat.}/K_m$ values for the *Strep. cacaoi* β-lactamase are consistently much lower for the cephalosporins, but conversely cephaloridine, cephalothin, cephacetrile, cephaloglycin, cefazolin and cefamandole are excellent substrates of the *Actinomadura* R39 β-lactamase, better than carbenicillin and ticarcillin. The

Actinomadura R39	G	D	K	T	G	G	G
Strep. albus G	G	D	K	T	G	A	G
Strep. cacaoi	E	D	K	S	G	Q	V
B. licheniformis	A	D	K	T	G	A	A
RTEM-1,2	A	D	K	S	G	A	G
RTEM-3	A	D	K	S	G	A	S
Staph. aureus	A	D	K	S	G	Q	A
Consensus class A		D	K	T/S	G		
Consensus class C	V	H	K	T	G	A/S	T

Fig. 6. Sequences around the conserved 'KTG' triad of β-lactamases

The RTEM-3 enzyme differs from RTEM-2 only by substitutions at residues 104 and 238 [Ambler's (1980) numbering]. It has a strongly increased activity against cefotaxime (Sougakoff *et al.*, 1988). K is residue 234 in Ambler's (1980) numbering.

relatively high efficiency of the same enzyme against cefuroxime and cefotaxime is also noteworthy, and we have already mentioned the fact that for the same oxyiminocephalosporins the $k_{cat.}$ values are consistently much higher with class A enzymes than with class C.

It is thus quite dangerous to consider class A β-lactamases indiscriminately as poor cephalosporinases. In fact, with some substrates, they are better cephalosporinases than class C enzymes!

K_m values

Like the other parameters, the K_m values for class A enzymes exhibit a very wide range of variation. Two of the most striking examples are carbenicillin (> 10 mM with the *Strep. albus* G enzyme and 8 μM with the RTEM-2 enzyme) and cephacetrile (> 6 mM with the *Strep. albus* G enzyme and 10 μM with the *B. licheniformis* enzyme). Each enzyme, however, appears to present a more coherent pattern: with the *Strep. albus* G enzyme the K_m values are always large, none of the values being below 200 μM, and many being larger than 1 mM. The behaviour of the *Strep. cacaoi* enzyme is similar, but the K_m values are distinctly lower with many penicillins. The results obtained with the two *Streptomyces* enzymes, particularly with the *Strep. albus* G β-lactamase, suggest that high K' values might be general properties of those enzymes. The

K_m values of the *Actinomadura* R39 β-lactamase are somewhat lower, around 100 μM, although values larger than 1 mM are still observed with some cephalosporins. The majority of the K_m values for the *B. licheniformis* β-lactamase are below 100 μM, but three values larger than 1 mM are observed. Martin & Waley (1988) have measured the individual values of k_{+2} and k_{+3} for β-lactamase I of *B. cereus* and found that acylation and deacylation occurred at similar rates for several substrates. The kinetic parameters of the *B. licheniformis* and *Actinomadura* R39 β-lactamases towards many penicillins are very similar to those of β-lactamase I of *B. cereus*, and one is tempted to conclude that the individual rate constants are also similar for the three enzymes. With some cephalosporins, however, such as ceftazidime and cefotaxime, the behaviours of the *B. licheniformis* and *Actinomadura* R39 enzymes seem to be closer to those of the *Streptomyces* β-lactamases, with high K' values. The RTEM-2 enzyme, finally, probably exhibits the most clear-cut pattern: the K_m values are low with all penicillins and high with the cephalosporins, if one excepts nitrocefin. With that enzyme the values of k_{+2} and k_{+3} for benzylpenicillin are also similar (S. G. Waley, personal communication), and the low K_m reflects a low K' value. Some penicillins also exhibit low K_m values with the *Staph. aureus* enzyme, and the same situation (similar k_{+2} and k_{+3} values) appears to prevail (S. G. Waley, personal communication), implying K' values in the micromolar range, although the results obtained by S. G. Waley are not in agreement with those reported by Pratt *et al.* (1988), who concluded that deacylation was rate-limiting in the hydrolysis of benzylpenicillin by the *Staph. aureus* enzyme. In contrast, the K_m values of the same enzyme for cloxacillin, methicillin, cephaloridine and cephalosporin C are significantly higher, indicating high K' values.

This high degree of diversity in class A again contrasts with the behaviour of class C, where most K_m values are below 100 μM, with the striking exceptions of 7-aminocephalosporanic acid, 7-aminodeacetoxycephalosporanic acid and cefazolin. At the present stage it remains difficult to draw firm conclusions from those comparisons, but it can safely be assumed that the large K_m values reflect high K' values. In class C, low K_m values often (or always) reflect rate-limiting deacylation, but this is certainly not true in class A. The relative contributions of K', k_{+2} and k_{+3} to the K_m values must await the determination of the individual values of those parameters, which still presents many experimental difficulties, but the analysis of the available data demonstrates that one should expect a wide dispersion of the K' values of class A β-lactamases.

Residues following the 'KTG box'

Most serine penicillin-recognizing enzymes sequenced so far contain a Lys-Thr-Gly sequence between the active-site serine residue and the *C*-terminus. In the *Streptomyces* R61 extracellular DD-peptidase lysine is replaced by histidine, and in some class A β-lactamases threonine is replaced by serine. However, in all enzymes whose three-dimensional structure has been studied, those three residues are part of the external strand of a β-pleated sheet adjacent to the substrate-binding pocket. Various mutations in the two residues that follow this (nearly) conserved triad have been shown to modify the specificity of the enzymes. The sequences around the triad of the enzymes studied here is shown in Fig. 6. It is quite clear that the variations of the two residues that follow the triad fail to explain the similarities and the differences in the specificity profiles. In particular, the identical alanine and glycine residues in the RTEM-2 and *Strep. albus* G enzymes are associated with enzymes of extremely different behaviour. Similarly, the glutamine residue found in both the *Strep. cacaoi* and the *Staph. aureus* enzymes cannot be correlated with similar specificities. One must conclude that other residues around the binding pocket also play a key role in determining the substrate profiles of the enzymes, although the residues following the 'KTG box' certainly contribute to those properties.

Thermal stability

Again the results presented in Table 10 indicate a clear contrast between the homogeneity of class C and the diversity of class A. Strikingly, the *Actinomadura* R39 β-lactamase is the most unstable of all, whereas the *B. licheniformis* enzyme is significantly more stable than all class C enzymes. Surprisingly, that pair of enzymes had obtained the highest score in the sequence comparisons! Two additional points are worth noting. (i) As observed earlier by Schultz *et al.* (1987) with the RTEM-1 β-lactamase, the enzymes were more stable at low than at high concentrations. Although the differences were not spectacular, this was rather unexpected and remains unexplained. If specific bimolecular interactions were playing a role in this phenomenon, one would expect the concentration effect to be much more drastic. (ii) Inactivation of the P99 β-lactamase did not obey first-order kinetics. There are only four amino acid substitutions between the P99 and 908R β-lactamases (Galleni *et al.*, 1988*a*), and they did not appear to influence any of the properties of the enzymes, if one excepts the space groups in which the two proteins crystallize. A reasonable hypothesis would be that the substitutions confer rather different surface properties to the two proteins.

Lags and bursts

Bursts, first described with a class A enzyme by Citri *et al.* (1976), appear to be rather common phenomena with β-lactamases (Joris *et al.*, 1986; Amicosante *et al.*, 1988; Galleni *et al.*, 1988*b*). These phenomena can be interpreted by assuming that the acyl-enzyme can slowly isomerize into an inactive or less active structure. In various cases, however, we also observed lags with methicillin, cloxacillin or oxacillin. To our knowledge, it is the first time that such a result has been recorded for β-lactamases. These observations, suggesting the isomerization of the acyl-enzyme into a more active form, certainly deserve further investigation.

CONCLUSIONS

It is quite safe to conclude that the diversity of the properties of class A β-lactamases will make the task quite difficult for anybody who attempts to divide them into sub-classes. This is made even more complicated by the fact that, quite often, the data found in the literature are unreliable or incomplete. Bush (1989*a*,*b*,*c*) has recently performed a very careful and thorough survey of the properties of these enzymes and proposed a new classification based on the substrate profiles and the inhibition by various β-lactams, EDTA and *p*-chloro-

mercuribenzoate. On the basis of the available data, Bush (1989*a*,*b*,*c*) has distinguished 'penicillin-hydrolysing β-lactamases (Pen-Y, group 2a)' from 'broad spectrum β-lactamases (BDS-Y, group 2b)', and included the three Actinomycetes, the *Staph. aureus* and the *B. licheniformis* enzymes in the first group and the RTEM-2 and the *C. diversus* enzymes in the second. Our study, which involves a large number of compounds, demonstrates that the 'spectrum' of the *B. licheniformis* and Actinomycetes β-lactamases might be just as 'broad' as those of the TEM and *C. diversus* enzymes!

As already discussed (Galleni *et al.*, 1988*b*), the most usually measured value is unfortunately the 'relative rate of hydrolysis' at a given β-lactam concentration. Depending upon the K_m value, this 'relative rate' can represent an estimation of either $k_{cat.}$ or $k_{cat.}/K_m$ and its widespread utilization does not simplify the problem. The sensitivity to *p*-chloromercuribenzoate might also be a misleading factor: in some experiments the concentrations were so large that the specificity of the reagent can be considered as doubtful. In addition, various enzymes contain a cysteine residue next to the active-site serine residue, and the inactivation by *p*-chloromercuribenzoate might only reflect steric hindrance.

The substrate profile might also be significantly altered by a very small number of residue substitutions. This is exemplified by the RTEM-2 and RTEM-3 β-lactamases, where two substitutions confer a vastly increased activity against cefotaxime and ceftazidime to the latter enzyme. This demonstrates the difficulty (if not the impossibility) of establishing classification criteria that would reconcile the structural and catalytic aspects.

Although we do not wish to sound pessimistic, we believe that, when carefully determined, the catalytic properties of class A β-lactamases will present a continuum, where only the extremes will fall into clearly distinct groups. Any classification based on a well-chosen number of β-lactams will be liable to modifications upon addition of new compounds. High-resolution three-dimensional structures of three of the enzymes discussed above (those of *Strep. albus* G, *B. licheniformis* and *Staph. aureus*) will probably become available in the near future. Those results will certainly help us to understand the specificity profiles of those enzymes, but it is quite doubtful that they will bring any definite solution to the classification problem.

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