Eur. J. Biochem. 217, 61–67 (1993)

Interactions between active-site serine β-lactamases and so-called β-lactamase-stable antibiotics

Kinetic and molecular modelling studies

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(Received May 14/June 29, 1993) – EJB 93 0706/3

The interactions between imipenem and four monobactams and three class A β-lactamases have been studied in detail. Despite their reputation as being β-lactamase-stable, some of these compounds were significantly hydrolysed by the enzymes. The results obtained with the *Streptomyces albus* G β-lactamase have been analysed in the light of molecular modelling studies. The discussion is extended to include other so-called β-lactamase-stable antibiotics to demonstrate that this appellation can often be misleading.

β-Lactamases generally catalyse the hydrolysis of the β-lactam ring amide bond in penicillins and related antibiotics with high efficiency. This reaction gives rise to biologically inactive products and represents the most widespread mechanism of resistance that bacteria have devised to escape the lethal action of this family of compounds (for reviews, see Frère et al., 1991; Waley, 1992).

In the fight against β-lactamase-producing pathogenic bacteria, one of the strategies has been to search for, and use, β-lactam compounds that are resistant to the hydrolytic action of these enzymes while retaining antibacterial activity (Knowles, 1985). In the light of the well established similarity between serine β-lactamases, which constitute the large majority of the penicillin-destroying enzymes, and the penicillin-sensitive DD-peptidases (Joris et al., 1988; Ghuysen, 1991), finding compounds which efficiently acylate the latter while remaining unconcerned by the potent hydrolytic activity of the β-lactamases may actually appear as an impossible mission. Nevertheless, several families of β-lactams are presently available which have acquired the reputation of being β-lactamase-stable.

Third-generation cephalosporins (e.g. cefotaxime and ceftazidime) represent one such group, in addition to cephamycins (cefoxitin), oxacephamycins (moxalactam), 6α-methoxypenams (temocillin), monobactams (aztreonam) and carbapenems (imipenem). This stability is however neither absolute nor general. Most of the cited compounds behave as sluggish substrates of the most current β-lactamases produced by pathogenic strains (the SHV, TEM and class C β-lactamases) and some little studied enzymes, often synthesized by more exotic strains, have been found to hydrolyse one or several of these β-lactams with various degrees of efficiency (Bush, 1989; Franceschini et al., 1993). Moreover, selective pressure has resulted in the emergence of point mutations which have considerably increased the specific activity of the SHV and TEM enzymes against compounds such as cefotaxime, ceftazidime, aztreonam and even imipenem (Philippon et al., 1989; Jacoby and Medeiros, 1991; Payne and Amyes, 1991). In consequence, it now appears of prime importance to explore how the slight structural variations between the different active-site serine β-lactamases can explain these modified kinetic characteristics. Conversely, a valuable complementary approach consists in the careful and detailed study of the interactions between one or several enzymes and a representative set of potential substrates. For instance, we have shown in a previous contribution (Matagne et al., 1990) that the sensitivity of several third-generation cephalosporins was far from negligible when exposed to the β-lactamases produced by *Actinomadura* R39, *Streptomyces albus* G and *Streptomyces cacaoi*.

In the present article, we extend the study of the catalytic profiles of these three class A β-lactamases to monobactams and imipenem (Fig. 1), compounds usually considered as being β-lactamase-stable, and we present a molecular modelling attempt to correlate the kinetic data with the structures of the Henri-Michaelis complexes that these compounds form with the *S. albus* G enzyme, whose structure is known at 0.17 nm (Dideberg et al., unpublished results).

Monobactams, originally discovered as natural compounds produced by bacteria (Imada et al., 1981; Sykes et al., 1981), are monocyclic compounds where the carboxylate group of classical β-lactams is replaced by a sulphonate moiety on N1. This electron-withdrawing substituent activates simple monocyclic β-lactams, which are very weak antibiotics, and also provides the negative charge required for recognition by DD-peptidases and β-lactamases (Gordon et al., 1982; Varetto et al., 1991). Aztreonam (SQ 26,776), the first synthetic monobactam to be developed as an antimicrobial agent (Sykes et al., 1982), and the other monobactams studied here are characterized by 3β-aminothiazolyl oxime

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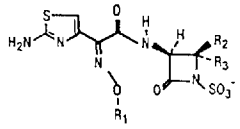
Abbreviations. SHV and TEM, plasmid-encoded class A β-lactamases.

Enzymes. β-Lactamases from *Streptomyces albus* G, *Streptomyces cacaoi* and *Actinomadura* R39 (EC 3.5.2.6).

Monobactams



	R_1	R_2	R_3
SQ 81.402	$C(CH_3)_2CO_2^-$	H	H
SQ 26.917	$C(CH_3)_2CO_2^-$	CH_3	H
Aztreonam	$C(CH_3)_2CO_2^-$	H	CH_3
Carumonam	$CH_2\text{-}CO_2^-$	CH_2OCONH_2	H

Imipenem (*N*-formimidoylthienamycin)

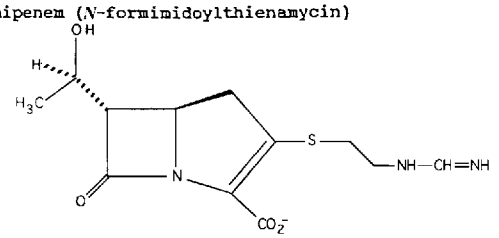


Fig. 1. Structures of the studied molecules.

side chains which are considered to improve their stability towards β-lactamases (Bush et al., 1982).

Imipenem (*N*-formimidoylthienamycin), a carbapenem, is a semisynthetic derivative of thienamycin. It is a broad-spectrum antibiotic (Fisher, 1984), generally regarded as β-lactamase-resistant (Toda et al., 1980; Kropp, 1985; Labia et al., 1986).

MATERIALS AND METHODS

Enzymes

Actinomadura R39, *Streptomyces albus* G and *Streptomyces cacaoi* β-lactamase preparations were those described before (Matagne et al., 1990).

β-Lactam compounds

Aztreonam, SQ 81804 and SQ 26917 were from the Squibb Institute for Medical Research, carumonam was from Hoffman-LaRoche and imipenem was from Merck, Sharp and Dohme Research Laboratories. All these compounds were kindly given by the respective companies. Nitrocefin was purchased from Oxoid. The structures of the various compounds are shown in Fig. 1.

Kinetic model

Hydrolysis of β-lactam antibiotics by serine β-lactamases can be generally described by a simple scheme involving a non-covalent (EC) and an acyl-enzyme (EC*) intermediates

$$E + C \underset{k_{-1}}{\overset{k_{-1}}{\rightleftharpoons}} EC \xrightarrow{k_2} EC^* \xrightarrow{k_3} E + P \quad \text{(model 1a)}$$

where k_2 and k_3 are first-order rate constants, respectively for acylation and deacylation. In most cases, the reaction can be quantitatively described by the Henri-Michaelis equation and the steady-state parameters are

$$k_{cat} = k_2 \times k_3/(k_2 + k_3), \quad (1)$$

$$K_m = k_3 \times K'/(k_2 + k_3) \quad (2)$$

where

$$K' = (k_{-1} + k_2)/k_{+1}, \quad (3)$$

and

$$k_{cat}/K_m = k_2/K'. \quad (4)$$

Eqn (4) shows that the apparent second-order rate constant for substrate hydrolysis (k_{cat}/K_m) corresponds to the apparent second-order rate constant for acyl-enzyme formation (k_2/K').

If k_3 is very low or equal to zero, C becomes a transient or irreversible inactivator (model 1b). Similarly, if $k_2 = 0$, C becomes a competitive inhibitor (model 1c).

Determination of the kinetic parameters

Usually, the steady-state parameters (k_{cat}, K_m and k_{cat}/K_m) were derived from the analysis of complete hydrolysis time courses (De Meester et al., 1987). When the K_m value was too high, only the k_{cat}/K_m ratio could be determined. This was performed by determining initial rates (v_0) at substrate concentrations ([C]) yielding constant $v_0/[C]$ ratios ([C] $\ll K_m$). Hydrolysis of the various compounds was directly recorded at 320 nm (monobactams) or 300 nm (imipenem). Conversely, when the K_m value was very low, it was measured as a K_i with 100 μM nitrocefin as substrate (Matagne et al., 1990). The k_{cat} value was then derived from initial rates at saturating substrate concentrations ([C] $\gg K_m$).

Progressive inactivation of the enzyme was monitored continuously with the help of the reporter substrate method (De Meester et al., 1987). Analysis of the reporter substrate hydrolysis time course yielded a pseudo-first-order inactivation rate constant

$$k_i = k_3 + k_2 \times [C]/(K' \times \alpha + [C]) \quad (5)$$

where α is a correction factor accounting for the protection of the enzyme by the reporter substrate S and

$$\alpha = 1 + [S]/K_{m,S}$$

where $K_{m,S}$ is the K_m of the reporter substrate.

When k_3 was non-negligible, the reporter substrate utilization eventually reached a steady state (v_{ss}) and the K_m for the studied compound C ($K_{m,C}$) could also be computed with the help of Eqn (6) which accounts for the competition between the two substrates

$$v_0/v_{ss} = 1 + [K_{m,S}/(K_{m,S} + [S])] \times [C]/K_{m,C} \quad (6)$$

where v_0 was the initial rate of hydrolysis of the reporter substrate in the absence of C.

Experimental conditions

All experiments were performed at 30°C in 50 mM sodium phosphate, pH 7.0. Dilution of the enzymes below a

Table 1. Kinetic parameters for the interaction between the three enzymes and monobactams.

Monobactam	*Actinomadura* R39			*Streptomyces albus* G			*Streptomyces cacaoi*		
	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m
	μM	s^{-1}	$M^{-1}\ s^{-1}$	μM	s^{-1}	$M^{-1}\ s^{-1}$	μM	s^{-1}	$M^{-1}\ s^{-1}$
Aztreonam	270 ± 10	20 ± 1	76 000 ± 4000	>1000	> 0.5	400 ± 50	1250 ± 50	165 ± 5	130 000 ± 15 000
SQ 81 402	> 10 000	> 1000	34 000 ± 3000	> 1000	> 3	2600 ± 300	600	95	160 000 ± 10 000
SQ 26 917	> 500	> 6	12 000 ± 1000	> 200	> 0.001	4 ± 0.5	> 5000	> 5	1 000 ± 150
Carumonam	> 1000	< 0.3	< 300	> 1000	> 0.005	6 ± 2	> 1000	< 0.2	< 200

Table 2. Kinetic parameters for the interaction between the three enzymes and imipenem. The methods utilized were (A) reporter substrate and (C) direct hydrolysis.

Parameter	Value (method) for		
	A. R39	*S. albus* G	*S. cacaoi*
k_2	$(1.9 \pm 0.2) \times 10^{-3}\ s^{-1}$ (A)	$k_{cat} = 0.04 \pm 0.007\ s^{-1}$ (C)	$> 0.14\ s^{-1}$ (A)
K'	(0.22 ± 0.01) μM (A)	$K_m = 0.12 \pm 0.015$ mM (C)	> 0.4 mM (A)
k_2/K'	9000 ± 1000 $M^{-1}\ s^{-1}$ (A)	320 ± 20 $M^{-1}\ s^{-1}$ (C)	360 ± 20 $M^{-1}\ s^{-1}$ (A)
k_3	$< 10^{-4}\ s^{-1}$ (C)	–	$2.5 \times 10^{-3}\ s^{-1}$ (A)
Conclusion	inactivator	poor substrate	very poor substrate/ poor transient inactivator

concentration of 0.1 mg/ml was performed with buffer solutions containing 0.1 mg/ml of bovine serum albumin.

Nitrocefin was used as reporter substrate in all experiments. The absorbance variations were followed at 482 nm and the conditions were chosen so that its utilization remained below 10%.

The concentration of nitrocefin (100 μM) was such that the correction factor α was negligible for the β-lactamases of *S. albus* G ($K_{m,S} = 1100 \pm 140$ μM) and *S. cacaoi* ($K_{m,S} = 1300 \pm 120$ μM) and was ≈2.4 for the β-lactamase of *A.* R39 ($K_{m,S} = 70 \pm 5$ μM), so that no large errors were introduced (De Meester et al., 1987).

Absorbance measurements were performed with the help of microcomputer-linked Beckman DU-8 or Uvikon 860 spectrophotometers.

Molecular modelling

The structures of the β-lactam compounds were optimized by the AM1 semi-empirical method (Dewar et al., 1985). The molecules were docked into the active site of the *S. albus* G enzyme and the energy of the Henri-Michaelis complexes thus obtained was minimized as described by Lamotte-Brasseur et al. (1991).

RESULTS

Of the four monobactams under study, only carumonam could apparently be considered as β-lactamase-stable (Table 1). The three other compounds were significantly hydrolysed but SQ 26917 exhibited a somewhat higher resistance to the *S. cacaoi* and *A.* R39 enzymes and was barely recognized by the *S. albus* G β-lactamase.

Conversely, imipenem was a poor substrate of the *S. albus* G and a very poor substrate of the *S. cacaoi* enzyme. It behaved as a rather efficient inactivator of the *A.* R39 β-lactamase, with which no turn-over was observed (Table 2).

The most striking results of the molecular modelling studies are summarized in Table 3. The listed distances are those computed at the level of the Henri-Michaelis complex obtained by docking the various compounds in the active site of the *S. albus* G enzyme. For comparison purposes, the table also shows the corresponding values (Lamotte-Brasseur et al., 1991) for a very good (benzylpenicillin), a fair (cephalosporin C) and several poor or very poor substrates. For each compound, log (k_2/K'), characterizing the rate of acylation of the active-site serine side chain is also displayed. A simplified scheme of the situation in the enzyme active site is given in Fig. 2.

DISCUSSION

It is interesting to analyse the results presented here in the broad perspective of the properties of compounds usually presented as being β-lactamase-stable. The interactions between some of these and β-lactamases can be depicted by models 1a, 1b and 1c presented above. Alternatively, with some β-lactams, model 2a seems to prevail, where EC** is a second acyl-enzyme, in which the antibiotic moiety has rearranged. With some cephalosporin-like compounds, this rearrangement involves the elimination of the leaving group on C3′ (Faraci and Pratt, 1985):

$$E + C \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EC \xrightarrow{k_2} EC^* \xrightarrow{k_3} E + P \quad \text{(model 2a)}$$

$$\downarrow k_4$$

$$EC^{**} \xrightarrow{k_5} E + P'.$$

When $k_3 \ll k_4$ or $k_3 = 0$, all the reaction flux is channelled through EC** (model 2′a) and, in addition, if $k_5 = 0$, the compound behaves as an irreversible inactivator (model 2b). With β-halogenopenicillanates (Frère et al., 1982; Knowles, 1985), k_3 can be larger than k_4 and k_5 negligible (model 2d). Finally,

Table 3. Principal features of the free enzyme and H-M complexes as calculated by molecular modelling. The distances listed in the last three columns are shown as dashed lines in Fig. 2.

Compound	$\log[(k_2/K')/M^{-1}\,s^{-1}]$	Distance between			
		Oγ Ser70–C1	HOγ Ser70–OW1	O1Glu 166–HW1	O2Glu 166–HW1
		nm			
Benzylpenicillin	6.45	0.288	0.207	0.170	0.219
Cephalosporin C	4.6	0.286	0.206	0.169	0.228
SQ81402	3.42	0.302	0.229	0.167	0.227
Cefotaxime	3.0	0.290	0.213	0.210	0.173
Aztreonam	2.6	0.318	0.255	0.251	0.167
SQ26917	0.54	0.311	0.255	0.251	0.165
Thienamycin	2.52	0.306	0.282	0.277	0.167
Ceftazidime	< 0	0.293	0.360	0.223	0.170
Moxalactam	<−1.5	0.304	0.750	0.347	0.168
Cefoxitin	<−1.6	0.292	0.750	0.339	0.167
Free enzyme	–	–	0.186	0.170	0.219

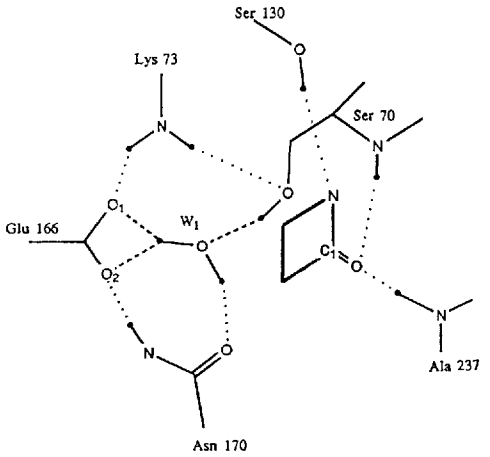


Fig. 2. Schematic representation of a β-lactam (heavy lines) in the active site of the *S. albus* G β-lactamase. Some of the important hydrogen bonds are shown as dotted lines (···). The three hydrogen bonds described in Table 3 and discussed in the text are represented as heavier dashed lines (----). Not all the hydrogen atoms are shown.

with clavulanate and sulbactam, a more complex pathway, involving one additional branch leading to an irreversibly inactivated enzyme, has also been encountered (Knowles, 1985). These two latter cases will not be further discussed here.

The results obtained with imipenem in the present study can be interpreted on the basis of models 1a for the *S. albus* G and *S. cacaoi* and 1b for the *A.* R39 enzymes. However, Monks and Waley (1988) and Zafaralla and Mobashery (1992) have shown that model 2a probably prevailed with two other class A β-lactamases (β-lactamase I of *Bacillus cereus* and TEM-1); it remains possible that the acyl-enzyme might also rearrange in the interactions described here, but this reaction would be fast, so that the global steady state would be established within the manual mixing dead time of a few seconds.

No such rearrangement can be expected with the monobactams and their interactions with the studied enzymes obeyed type 1 models. There was little or no activity versus carumonam, and, in the other cases, the K_m values were large and no accumulation of acyl-enzyme could be detected. The rather high k_{cat}/K_m and k_{cat} values observed in several cases should be underlined (Table 1).

All these results are in sharp contrast with the behaviour of class C β-lactamases, with which imipenem, aztreonam and carumonam efficiently form stable acyl-enzymes with k_2/K' values ranging over $(1-30\times10^4)$ $M^{-1}\,s^{-1}$ and very low k_3 values $[(0.2-30)\times10^{-3}\ s^{-1}]$, which results in K_m values lower than 1 μM (Galleni et al., 1988). Only the *S. cacaoi* β-lactamase–imipenem interaction would exhibit a similarly low K_m value (6 μM) but the efficiency of the acylation reaction (360 $M^{-1}\,s^{-1}$) is significantly lower. It is also interesting to note that a class A enzyme, produced by *Pseudomonas stutzeri*, has been shown to exhibit high k_{cat}/K_m values with aztreonam and carumonam, but these are accompanied by rather high k_{cat} values ($3-6\ s^{-1}$; Franceschini et al., 1993). Some TEM variants also efficiently hydrolyse aztreonam (Jacoby and Medeiros, 1991).

The kinetic parameters of the three Squibb monobactams can also be compared to those of ceftazidime (Table 4). The four compounds have the same acylamido side chain. The k_{cat}/K_m values are consistently lower with ceftazidime and SQ 26917. Surprisingly, the methyl group on C4 (corresponding to the C6 of cephalosporins) of the latter compound points to the β-face of the β-lactam ring and one would have expected the acylation by aztreonam, whose methyl group points to the α-face, to be more hindered by that geometry. Thus, with the class A β-lactamases tested here, the hydrolysis of carumonam and SQ 26917 (*cis* configuration) is significantly impeded compared to that of aztreonam (*trans* configuration), a situation consistent with the findings of Bush et al. (1982) but which firmly contrasts with that found with the β-lactamase of *Morganella morganii* (class C), which inactivates more rapidly monobactams with a 3,4-*cis* configuration (Matsuda et al., 1991).

Cephalosporins exhibiting 7-β side chains containing a similar oximino group (cefuroxime, cefmenoxime, cefotaxime, ceftazidime, ceftizoxime and ceftriaxone) were initially considered as being β-lactamase-stable mostly on the basis of studies performed with class C and a limited number of class A (TEM-1/2, SHV-1, *S. aureus*) β-lactamases. Some of these compounds are included in Table 5, together with cefoxitin, moxalactam and temocillin in which a methoxy group is found on the α-face of the β-lactam ring. This table

Table 4. k_{cat}/K_m values of class A β-lactamases for monobactams and for ceftazidime. Data in the last two columns were obtained by Bush et al. (1982).

Compound	k_{cat}/K_m for				
	A. R39	*S. albus* G	*S. cacaoi*	TEM-2	*K. pneumoniae*
	$M^{-1}\ s^{-1}$				
Aztreonam	76 000	400	130 000	1 800	210 000
SQ 81 402	34 000	2600	160 000	130 000	740 000
SQ 26 917	12 000	4	1 000	52	1 400
Ceftazidime	13 000	<1	3 500	270	130

Table 5. Overview of the interaction between so-called β-lactam-stable compounds and β-lactamases of classes A and C. The first figure refers to the relevant scheme(s). It is not always easy to distinguish between schemes which only differ by the presence or absence of one branch (i.e. model 2′a is model 2a with $k_3 = 0$) and the data in the literature do not always present detailed analyses. The first letter refers to the value of k_{cat}/K_m or k_2/K': H (= high) $>10^5 M^{-1} s^{-1}$ > M (= medium) > $10^3 M^{-1} s^{-1}$ > L (= low) > $1 M^{-1} s^{-1} > 0$. The second letter refers to the rate of reactivation of the transiently inactivated enzyme: F (= fast) $>0.1\ s^{-1}$ > S (= slow) $>10^{-4}\ s^{-1} > 0$. In *some cases (hyd)*, the compound is a substrate for which the rate-limiting step has not been identified. The letter between round brackets then refers to the k_{cat} value (H > $10\ s^{-1}$ > M > $1\ s^{-1}$ > L > $0.1\ s^{-1}$ > VL). nd = not determined (no data). The references are: (1) Amicosante et al. (1988); (2) Bush et al. (1982); (3) Charnas and Then (1988); (4) Edmondson et al. (1981); (5) Edmondson and Reading (1985); (6) Faraci and Pratt (1985); (7) Faraci and Pratt (1986); (8) Fisher et al. (1980); (9) Franceschini et al. (1993); (10) Galleni et al. (1988); (11) Hashizume et al. (1984); (12) Jules and Neu (1982); (13) Labia (1982); (14) Labia et al. (1986); (15) Matagne et al. (1990); (16) Matagne et al. (1993); (17) Martin Villacorta et al. (1991); (18) Mazzella and Pratt (1989); (19) Monks and Waley (1988); (20) Murakami and Yoshida (1985); (21) Slocombe et al. (1981); (22) Zafaralla and Mobashery (1992).

Compounds	*A.* R39			*S. albus* G			*S. cacaoi*		
Cefoxitin	2b	L	O	1c	O	–	2′a	L	S
Moxalactam	2b	L	O	no interaction up to 1 mM			2a	L	S
Temocillin	1b	L	O	1c	O	–	1c	O	–
7β-Oximino cephalosporins									
cefotaxmine	2a	H	hyd(H)	2a	M	hyd(M)	2a	L	hyd(M)
ceftazidime	2a	M	hyd(H)	no interaction up to 1 mM			2a	M	hyd(M)
Monobactams									
aztreonam and SQ81402	1a	M	hyd(H)	1a	M/L	hyd(M)	1a	H	hyd(H)
SQ 26917	1a	M	hyd(H)	1a	L	hyd(VL)	1a	M	hyd(H)
carumonam	extremely poor substrate			extremely poor substrate			extremely poor substrate		
Imipenem	1b/2b	M	O	1a/2a	L	hyd(VL)	1a/2a	L	S

Compounds	Tem 1/2			Other class A			Class C			References
Cefoxitin	2′a	L	S	2′a/1c	L	S	2′a	H/M	F/S	7, 8, 10, 16–18
Moxalactam	little interaction slow inactivation[a]			little or no interaction[a]			2a	H	S	10, 13, 16, 18, 20
Temocillin	no hydrolysis detected			no hydrolysis detected except with β-lactamase I			no hydrolysis detected			4, 5, 12, 16, 17, 21
7β-Oximino cephalosporins										2, 3, 6, 9, 10, 15, 18
cefotaxime	2a	M	hyd(L)	2a	H/M	hyd (M/H)	2a	H	hyd (H/M/L/VL)	
ceftazidime	2a	L	hyd(VL)	2a	L/M	hyd (VL-M)	2a	M	hyd (VL)	
Monobactams										2, 9, 10
aztreonam and SQ81402	1a	H/M	hyd(M)	1a	H	hyd (H/M)	1a	H/M	S	
SQ26917	1a	L	hyd(VL)	1a	M	hyd(M)	stable but reversible complex			
carumonam		nd		1a	M	hyd(M)	1a	M	S	
Imipenem	2a	L	hyd(VL)	1a/2a	L/M	hyd (L/VL)	1a/2a	H/M	S	1, 9–11, 14, 19, 22

[a] Reported without quantitative details.

summarizes the results obtained with a large number of different β-lactamases but does not consider the TEM and SHV variants which were selected for their ability to hydrolyse third-generation cephalosporins (Sougakoff et al., 1988; Labia et al., 1988). Even when these variants are excluded, it can be seen that several of the original enzymes are perfectly capable of significantly hydrolysing some so-called β-lactamase-stable compounds. Some of these enzymes, when compared to the TEM and SHV variants, thus present an interesting case of converging evolution within the generally diverging class A proteins.

Even imipenem does not totally escape hydrolysis. Interestingly, the k_{cat} value observed with the *S. albus* G β-lactamase is quite similar to that determined with the R244S

mutant of the TEM-1 enzyme (k_{cat} = 0.04 s^{-1}; Zafaralla and Mobashery, 1992).

Thus, it seems that some class A enzymes are perfectly capable of hydrolysing so-called β-lactamase-stable antibiotics. Moreover, single point mutations can significantly increase the capacity of some inefficient enzymes to do so. Such a flexibility can be considered as somewhat alarming. Recently, the catalytic properties of class B β-lactamases, that require a zinc ion for activity (Zn^{2+} enzymes), were carefully analysed (Felici et al., 1993). It appeared that most of these enzymes hydrolysed, sometimes very efficiently (k_{cat}/K_m values up to $10^7 M^{-1} s^{-1}$), the studied β-lactamase-stable compounds (cefotaxime, cefoxitin, moxalactam and imipenem) but did not interact with aztreonam. Athougth these enzymes do not yet seem to represent an important clinical problem (Frère et al., 1991), they represent a potential danger and their catalytic properties towards so-called β-lactamase-stable compounds definitively render this appelation misleading.

How do the molecular modelling results correlate with the k_{cat}/K_m values observed with the *S. albus* G β-lactamase? The mechanism which has been proposed before underlines the important role of a water molecule (W1) which connects the side chains of Ser70, Glu166 and Asn170 and would serve as a relay in the activation of the Ser70 hydroxyl group by the Glu166 carboxylate acting as a general base (Lamotte-Brasseur et al., 1991). Breaking of the C-N β-lactam bond would subsequently involve the back delivery of the proton to the nitrogen atom via the Lys73 and Ser130 side chains. The data in Table 3 show that the distance between the serine Oγ and the β-lactam carbonyl carbon does not exhibit major variations but suggest a rough correlation between the HOγ Ser70−OW1 distances and the rate of enzyme acylation. Indeed, this distance is characteristic of a good hydrogen bond for the best substrates and becomes very large for the poorer substrates, increasing from 0.282 nm for thienamycin (or imipenem) to 0.75 nm for moxalactam and cefoxitin. With these two compounds, W1 has been completely displaced by the C7 α-methoxy group and the distance is so large that it becomes practically irrelevant. The situation with the three monobactams and cefotaxime is more complex and probably involves the distances between one of the W1 protons (the second is in interaction with the Asn170 side chain, whose role is thus important in the accurate positioning of W1) and the two oxygen atoms of the Glu166 carboxylate. With benzylpenicillin and cephalosporin C, this proton is significantly closer to O1 on which it can thus be easily transferred. In that case, back delivery of a proton to the β-lactam nitrogen via the Lys73 NH_3^+ group is thus facilitated. If the W1 proton is closer to Glu166 O2, it is preferentially transferred on this oxygen and back-delivery of a proton to the β-lactam nitrogen is impaired. This probably explains why SQ 81402 more readily acylates the enzyme than cefotaxime. Transfer of the W1 proton on Glu166 O2 is even more favoured with aztreonam which, together with the longer HOγ Ser70-W1O distance accounts for the fact that aztreonam and SQ 26917 are, in turn, poorer acylating agents than cefotaxime. It is also apparent that the data presented here do not supply a rational explanation for the differences between aztreonam and SQ 26917. The somewhat paradoxical situation with this pair of compounds has been underlined above.

One last point remains worth mentioning. Although SQ 26917 and ceftazidime appear generally to acylate class A β-lactamases much less efficiently than aztreonam and SQ 81402, all four compounds are rather well recognized by the *A.* R39 enzyme. This observation, together with other results described in this article, again stresses the wide diversity of the class A β-lactamases catalytic properties, which contrasts with the more uniform behaviour of the class C enzymes.

This work was supported, in part by the Belgian government in the frame of the *Pôles d'attraction interuniversitaires* (PAI 19), an *Action concertée* with the Belgian government (convention 89/94−130), the *Fonds de la recherche scientifique médicale* (contract 3.4537.88), and a *convention tripartite* between the *Région wallone*, Smith-Kline Beecham, UK, and the University of Liège.

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