Interactions between active-site-serine β-lactamases and mechanism-based inactivators: a kinetic study and an overview

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The interactions between three class A β-lactamases and three β-lactamase inactivators (clavulanic acid, sulbactam and olivanic acid MM13902) were studied. Interestingly, the interaction between the *Streptomyces cacaoi* β-lactamase and clavulanate indicated little irreversible inactivation. With sulbactam, irreversible inactivation was found to occur with the three studied enzymes, but no evidence for transiently inactivated adducts was found. Irreversible inactivation of the *S. albus* G and *S. cacaoi* enzymes was particularly slow. With olivanate, irreversible inactivation was also observed with the three enzymes, but with the *S. cacaoi* enzyme, no hydrolysis could be detected. A tentative summary of the results found in the literature is also presented (including 6β-halogenopenicillanates), and the general conclusions underline the diversity of the mechanisms and the wide variations of the rate constants observed when class A β-lactamases interact with β-lactamase inactivators, in agreement with the behaviours of the same enzymes towards their good and poor substrates.

INTRODUCTION

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 that family of compounds [for reviews, see Frère et al. (1991) and Waley (1992)].

Most β-lactamases contain a reactive serine hydroxy group at the active site (serine enzymes) and are divided in three classes (A, C and D) on a structural basis (Ambler, 1980; Jaurin and Grundström, 1981; Huovinen et al., 1988; Joris et al., 1991). Despite their structural diversity, all these enzymes catalyse β-lactam opening through the formation of an acyl-enzyme intermediate (Frère et al., 1991; Waley, 1992). Class B contains a small number of β-lactamases that require a zinc ion for activity (Zn^{2+} enzymes). Their mechanism of action is still poorly understood.

Since the first report in 1940 entitled 'An enzyme from bacteria able to destroy penicillin' (Abraham and Chain, 1940), enormous and continuing efforts have been devoted to obtaining molecules which would not be affected by the β-lactamases of pathogenic strains. So far two strategies have been developed to overcome the destructive action of β-lactamases. The first is based on the use of β-lactams that are resistant to the hydrolytic action of β-lactamases while retaining antibacterial activity. The second utilizes two β-lactams in synergy: one is a potent inactivator of the β-lactamase, but a poor antibiotic, while the other is a good antibiotic, but is sensitive to the β-lactamase action. The former protects the latter from rapid hydrolysis by the enzyme. The first strategy tends to avoid the enzyme, and the second neutralizes it (Knowles, 1985). The second strategy seems to be the most effective, since β-lactamase-resistant molecules were generally found to be poorer antibiotics (Knowles, 1985).

Although a few non-β-lactam inhibitors have been described, namely boronic acids (Kiener and Waley, 1978), most of the β-lactamase inhibitors are β-lactams. They can be largely considered as mechanism-based inhibitors (also known as 'suicide inhibitors' or 'suicide substrates') insofar as they are substrate-like reagents whose reactivity is strongly enhanced by a specific interaction with the enzyme, which can thus be considered as 'committing suicide'. Such a behaviour clearly distinguishes these compounds from active-site-directed inhibitors [for an exhaustive review on mechanism-based inhibitors, see Tipton (1989)]. The β-lactamase recognizes the inhibitor as a normal substrate and catalyses the opening of the β-lactam ring. The enzyme is then transiently or definitely inactivated as a stable covalent adduct (acyl-enzyme). Inhibition is an alternative to turnover (Cartwright and Waley, 1983). However, it must be stressed that, owing to the large diversity of β-lactamases, there are significant differences in the interaction of any given compound with different β-lactamases (Fink, 1985; Pratt, 1989). For example, penicillanic acid was found to be a good substrate for the β-lactamases from *Streptomyces albus* G and *Actinomadura* R39, while it behaved as an inactivator towards the *Streptomyces cacaoi* enzyme (Matagne et al., 1990). Moreover, the detailed mechanism by which a given β-lactamase is inhibited by various β-lactams can also be very different, even if the final result is the formation of a stable covalent complex at the acyl-enzyme stage or a derivative of that intermediate.

In other contributions (Matagne et al., 1993a, b), we describe the interactions between various class A β-lactamases and compounds which are usually considered as 'β-lactamase-stable' and which might thus reach their DD-peptidases targets while escaping the destructive activity of β-lactamases (first strategy). The present paper is devoted to compounds which have generally little antibiotic activity by themselves: they react with the DD-peptidases slowly or not at all. However, they behave as mechanism-based inactivators of several active-site-serine β-lactamases and are therefore able to potentiate the action of classical β-lactamase-sensitive compounds by protecting them from enzymic hydrolysis (second strategy). Here, we have analysed the interactions between clavulanic acid, sulbactam and an olivanic acid with the three class A β-lactamases produced by *Actinomadura* R39, *Streptomyces albus* G and *Streptomyces cacaoi*.

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MATERIALS AND METHODS

Enzymes

Actinomadura R39, *Streptomyces albus* G and *Streptomyces cacaoi* β-lactamase preparations were those used for the study of the substrate profiles (Matagne et al., 1990).

Compounds

Sulbactam was from Pfizer Central Research (Sandwich, Kent, U.K.), and clavulanic acid and olivanic acid MM13902 were from Beecham Pharmaceuticals (Brentford, Middx., U.K.). These compounds were kindly given by the respective companies. Nitrocefin was purchased from Oxoid (Basingstoke, Hants, U.K.). The structures of the various compounds are shown in Figure 1.

Determination of the kinetic parameters

β-Lactamases hydrolyse 'normal' substrates according to the linear pathway described by Model 1:

$$E+C \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EC \xrightarrow{k_2} EC^* \xrightarrow{k_3} E+P$$

Model 1

More complex interactions involve rearrangements of the initially formed acyl-enzyme EC* (Model 2a):

$$\begin{array}{l} E+C \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EC \xrightarrow{k_2} EC^* \xrightarrow{k_3} E+P \\ \qquad\qquad\quad \downarrow k_4 \\ \qquad\qquad\quad EC^{**} \xrightarrow{k_5} E+P' \end{array}$$

Model 2a

When $k_3 = 0$ or $k_3 \ll k_4$, C behaves as a substrate, and all the reaction flux is channelled through the second acyl-enzyme, EC** (Model 2a'). Irreversible inactivation of the enzyme occurs if $k_3 = k_5 = 0$ (Model 2b) or if $k_5 = 0$ (Model 2c).

Among the interactions which have been studied so far, those between clavulanic acid and class A enzymes (TEM-2, *Klebsiella pneumoniae* E70 and *Actinomadura* R39) appeared to be even more complex, involving a three-branch pathway, represented by Model 3:

$$\begin{array}{l} \qquad\qquad EC_i^* \\ \qquad\qquad \uparrow k_6 \\ E+C \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EC \xrightarrow{k_2} EC^* \xrightarrow{k_3} E+P \\ \qquad\qquad\quad \downarrow k_{+4} \quad \uparrow k_{-4} \\ \qquad\qquad EC^{**} \xrightarrow{k_5} E+P' \end{array}$$

Model 3

In this model, EC* might represent an already rearranged acyl-enzyme. The fate of the second (rearranged) acyl-enzyme, EC**, is not always clear, and it might undergo direct hydrolysis (k_5), back-rearrangement to EC* (k_{-4}) or both. It is usually impossible to distinguish between the k_{-4} and k_5 steps. Arbitrarily, the situation will be further analysed assuming that $k_{-4} = 0$.

Previous studies indicated that Model 2b was probably valid for the interaction between β-iodopenicillanate and the *S. cacaoi* β-lactamase (Lenzini and Frère, 1985) and Model 2c for the interaction between the same compound and the *Actinomadura* R39, *S. albus* G and *Bacillus licheniformis* enzymes (Frère et al., 1982a; De Meester et al., 1989). Models 2a and 2b were also encountered in the study of 'β-lactamase-stable' compounds (Matagne et al., 1993a,b).

In Model 2c, the ratio between the rate constants (k_3/k_4), Waley's (1980) partition ratio, represents the ratio between the number of productive turnovers and those leading to irreversible enzyme inactivation. It is determined from experiments where $[C]_0/[E]_0$ ($[C]_0$ and $[E]_0$ are the initial concentrations of inhibitor and enzyme respectively) is such that incomplete inactivation occurs. The remaining enzyme activity is measured for various $[C]_0/[E]_0$ values and the partition ratio deduced from the following relationship (Frère et al., 1982b; Tipton, 1989):

$$([E]_0 - [EC_i])/[E]_0 = 1 - [k_4/(k_3+k_4)] \cdot [C]_0/[E]_0 \qquad (1)$$

When, as observed in the present study, inactivation phenomena consistently obey pseudo-first-order time courses, it can be safely assumed that $k_2 \ll k_{-1}$ and the first step can be considered as a rapid equilibrium with $K = k_{-1}/k_{+1}$.

Providing that $k_3 \gg k_4$, conditions can then be chosen where one branch of the pathway has negligible effects on the other, and the values of $k_{cat.}$, K_m, k_2/K and $(k_i)_{lim}$ are computed as described previously (Frère et al., 1982b). Under conditions where hydrolysis can be neglected ($[C]_0/[E]_0 \gg k_3/k_4$), enzyme inactivation is characterized by k_i, a pseudo-first-order rate constant:

$$k_i = (k_i)_{lim} * [C]/([C]+K_m) \qquad (2)$$

where

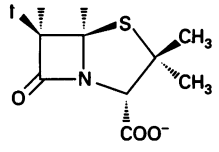
$$K_m = k_3 \cdot K/(k_2+k_3+k_4) \qquad (3)$$

and

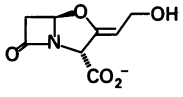
$$(k_i)_{lim} = k_2 \cdot k_4/(k_2+k_3+k_4) \qquad (4)$$

and thus

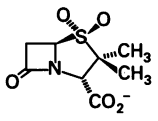
$$(k_i)_{lim}/K_m = k_2/K \cdot k_4/k_3 \qquad (5)$$



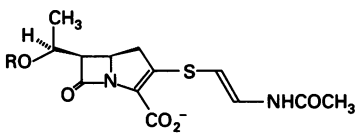
6β-Iodopenicillanic acid



Clavulanic acid



Sulbactam



Olivanic acids (epithienamycins)
MM 22382, R=H; MM 13902, R=SO_3^-

Figure 1 Structures of the studied molecules

and

$$k_{cat.}/(k_i)_{lim} = k_3/k_4 \quad (6)$$

where

$$k_{cat.} = k_2 \cdot k_3/(k_2+k_3+k_4) \quad (7)$$

Note that K_m and $k_{cat.}$ are nearly identical with those of the simple linear pathway if $k_3 \gg k_4$.

In this mechanism, $(k_i)_{lim}$ is a first-order rate constant characterizing the rate of the inactivation process at saturating inactivator concentrations. Inactivation rate constants (k_i) can be measured in the presence of a reporter substrate, as described by De Meester et al. (1987a), or directly, if the disappearance of the inactivator can be monitored. Steady-state parameters ($k_{cat.}$ and K_m) values are determined respectively by initial-rate measurements and competitive-inhibition experiments, performed rapidly, before the inactivation process becomes detectable (Frère et al., 1982b).

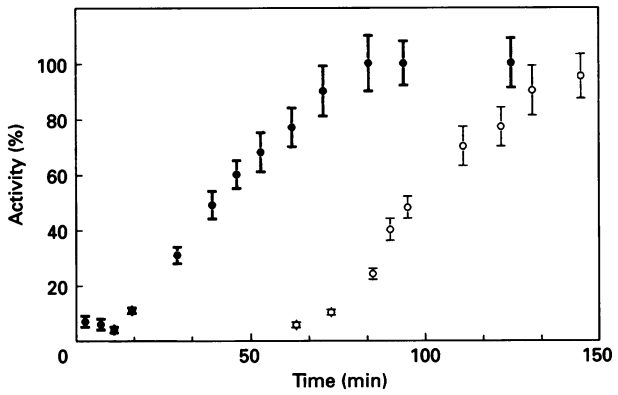


Figure 2 Transient inactivation of the *S. cacaoi* enzyme by clavulanate

Enzyme ($[E]_0 = 1.8\ \mu M$) was incubated with 5.5 (●) and 10 (○) molar proportions of inactivator. Samples were assayed for activity against 100 μM nitrocefin after various periods of time.

Experimental conditions

Kinetic experiments were all performed under the conditions described for the study of 'β-lactamase-stable' compounds (Matagne et al., 1993a,b). Hydrolysis of sulbactam and olivanic acid were recorded directly at 235 nm and 304 nm respectively.

RESULTS

Clavulanic acid

Clavulanic acid, a naturally occurring β-lactam, shows weak antibacterial activity, but displays potent β-lactamase inhibitory properties (Brown et al., 1976). In combination with amoxycillin (Augmentin), it represents a powerful clinical strategy to overcome the resistance of bacteria harbouring the TEM β-lactamase variants capable of hydrolysing third-generation cephalosporins (Seetulsingh et al., 1991). Yet a recent report (Thomson and Amyes, 1992) of a clinical isolate carrying a TEM-like enzyme that confers resistance to Augmentin (TRC-1 β-lactamase) will certainly disturb the confidence of clinicians.

Clavulanic acid is characterized by an oxazolidine ring, the absence of acylamino side chain and an unusual substituent on C-2.

The reactions of the β-lactamases of *Actinomadura* R39 and *S. albus* G with clavulanate proceed along branched pathways (Frère et al., 1982b). Conversely, a simple linear pathway has been proposed for the *S. cacaoi* enzyme (Ogawara and Mantoku, 1981). However, our results indicated a more complex interaction. At low $[C]_0/[E]_0$ ratios, a transient inactivation was observed, followed by a complete recovery of the enzyme activity (Figure 2). Longer incubations (24–48 h) with high $[C]_0/[E]_0$ ratios (up to 15000) resulted in a low level of irreversible inactivation; after exhaustive dialysis at 4 °C and a further 2 h incubation at 30 °C to re-activate any transiently inactivated enzyme, the activity was not completely recovered, but the irreversible loss of activity did not exceed 20–30 %. Although these results indicated the validity of Model 3, the value of k_6 was so low ($\leqslant 3 \times 10^{-5}\ s^{-1}$) as to make

Table 1 Kinetic parameters of β-lactamases for sulbactam

Notes: (a) measured directly under initial-rate conditions; (b) measured by monitoring the hydrolysis of sulbactam itself; (c) measured by the reporter substrate method; (d) calculated from the individual $k_{cat.}$ and $(k_i)_{lim}$ values; (e) measured directly in partial inactivation experiments (eqn. 1); (f) measured as K_i (*S. albus* G, *S. cacaoi*) or from the curves k_i versus [C] (*Actinomadura* R39); (g) and (h) calculated from individual values.

Parameter	*Actinomadura* R39	*S. albus* G	*S. cacaoi*	Note
$k_{cat.}$ (s^{-1})	13 ± 3	2 ± 0.1	0.022 ± 0.001	(a)
$(k_i)_{lim}$ (s^{-1})	$(5 \pm 0.3) \times 10^{-3}$	$(9 \pm 1) \times 10^{-5}$	$(6 \pm 1) \times 10^{-5}$	(b)
	$(8.3 \pm 0.05) \times 10^{-3}$	–	–	(c)
$\frac{k_{cat.}}{(k_i)_{lim}} \left(= \frac{k_3}{k_4}\right)$	1600–2600	22000	380	(d)
$\frac{k_3}{k_4}$	2800 ± 300	37000 ± 2000	400 ± 100	(e)
K_m (μM)	8.5 ± 1.5	4.5 ± 0.3	0.72 ± 0.04	(f)
$\frac{k_{cat.}}{K_m}$ ($M^{-1} \cdot s^{-1}$)	$(1.5 \pm 0.6) \times 10^6$	$(0.44 \pm 0.04) \times 10^6$	$(0.031 \pm 0.003) \times 10^6$	(g)
$\frac{(k_i)_{lim}}{K_m}$ ($M^{-1} \cdot s^{-1}$)	590–980	20 ± 4	80 ± 20	(h)

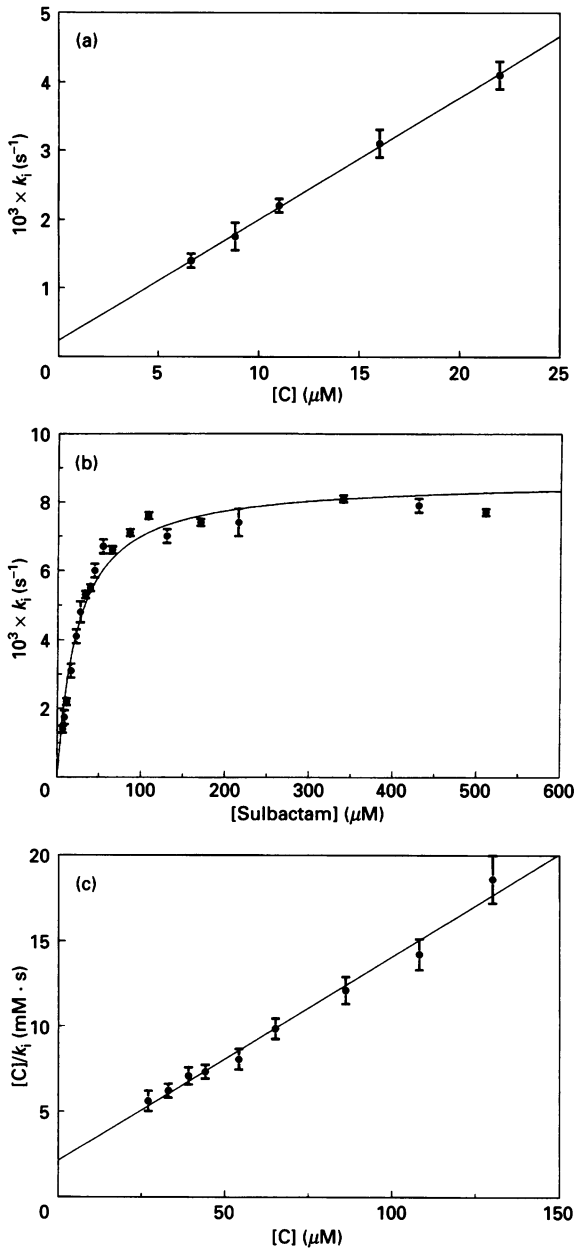


Figure 3 Interaction between the *Actinomadura* R39 β-lactamase and sulbactam

(**a**) Variation of k_i with [C] at low sulbactam concentrations from which a $(k_i)_{lim}/K_m$ value of 430 $M^{-1} \cdot s^{-1}$ could be obtained; (**b**) saturation phenomenon at larger concentrations [the data were fitted to eqn. (2)]; (**c**) Hanes plot of the data in (**b**) allowing the computation of $(k_i)_{lim} = 0.0084$ s^{-1} and $K_m = 7.2$ μM.

it irrelevant in *in vivo* situations, and Model 2a appears to be more relevant. A detailed kinetic analysis of that complex interaction is beyond the scope of the present study and remains the subject of further investigation.

Sulbactam (penicillanic acid sulphone)

Sulbactam (CP 45899), first reported by English et al. (1978), is obtained by oxidation of the thiazolidine sulphur of penicillanic acid and is used in combination with ampicillin for clinical application.

In contrast with results obtained, for instance, with the TEM-2 β-lactamase by Knowles and colleagues (Brenner and Knowles, 1981; Kemal and Knowles, 1981; Brenner and Knowles, 1984), which indicated the validity of Model 3, no evidence for transiently inactivated adducts was found with the three enzymes studied here and the experimental data were analysed on the basis of Model 2c, yielding the values shown in Table 1.

The partition ratio, k_3/k_4, was measured in partial-inactivation experiments with the help of eqn. (1). The partially inactivated samples, when left at 15 °C for 48 h, did not exhibit any activity recovery.

Directly monitoring the hydrolysis of sulbactam at 235 nm and at concentrations much larger than the K_m values and than $k_3[E]_0/k_4$, yielded the values of $k_{cat.}$ (from the initial rates) and of $(k_i)_{lim}$ from the progressive inactivation of the enzyme.

For the *S. albus* G and *S. cacaoi* enzymes, with which inactivation was quite slow, the K_m values were measured as K_i values using nitrocefin as substrate. With the *Actinomadura* R39 enzyme, the reporter-substrate method was used at $[C]_0/[E]_0 >$ 50000 and at low sulbactam concentrations, a $(k_i)_{lim}/K_m$ value of 450 ± 50 $M^{-1} \cdot s^{-1}$ was measured on the basis of the slope of the linear k_i-versus-[sulbactam] plot (Figure 3a). At higher concentrations, a hyperbolic dependency was observed from which the individual values of $(k_i)_{lim}$ ($(8.3 \pm 0.5) \times 10^{-3}$ s^{-1}) and K_m (7.2–9.7 μM) could be derived (Figures 3b and 3c), thus yielding a somewhat higher $(k_i)_{lim}/K_m$ value of 1000 ± 100 $M^{-1} \cdot s^{-1}$.

The hydrolysis of sulbactam by the *S. albus* G β-lactamase was also performed in the presence of 1 M NaCl, yielding a $k_{cat.}$ of 1.6 ± 0.1 and a $(k_i)_{lim}$ of about 3×10^{-5} s^{-1}. Thus the partition ratio was not dramatically altered by the presence of high salt concentrations, in contrast with the results obtained with the same enzyme and β-halogenopenicillanates (De Meester et al., 1989).

Olivanic acid MM13902 [(5*R*,6*R*)-*cis*-carbapenem]

Olivanate MM13902 (epithienamycin E; R = SO_3^-) is one of the first carbapenems isolated (Brown et al., 1977). It is characterized by a sulphate moiety on the 6β-(α-hydroxyethyl) side chain. The importance of this group was underlined by the fact that the analogous compound, MM22382, lacking the sulphate group (epithienamycin D; R = H), behaved as a good substrate for the TEM enzyme and was a relatively inefficient inactivator (Charnas and Knowles, 1981a; Easton and Knowles, 1982).

The *S. albus* G β-lactamase hydrolysed the olivanic acid, but progressive inactivation occurred. Inactivation was complete with $[C]_0/[E]_0 > 1000$. No re-activation of the partially inactivated samples was observed after 24 h at 20 °C. The initial rates of hydrolysis were independent of olivanate concentrations at $[C]_0 = 50$ μM and yielded a $k_{cat.}$ value of 0.4 ± 0.04 s^{-1}. Under the same conditions, the rate of progressive inactivation (measured either directly or in the presence of a reporter substrate) was similarly independent of the inactivator concentration, and yielded a $(k_i)_{lim}$ value of $(3 \pm 1) \times 10^{-3}$ s^{-1}. These data were in good agreement with Model 2c, but competitions with nitrocefin, performed under initial-rate conditions, suggested a K_m ($= K_i$) value of 14 μM. In disagreement with this value, the apparent first-order rate constant for inactivation (k_i) remained independent of $[C]_0$ in the 8–20 μM range.

Qualitatively, the *Actinomadura* R39 β-lactamase behaved as the *S. albus* G enzyme, a $[C]_0/[E]_0$ value of 100 yielding partial inactivation. A $k_{cat.}$ value of 0.7 ± 0.1 s^{-1} was observed under initial-rate conditions ($[C]_0 = 50$ μM) and a k_i value of

$(8 \pm 2) \cdot 10^{-3}$ s^{-1} was derived from the rate of progressive inactivation for $[C]_0 = 100$ μM.

Conversely, the *S. cacaoi* β-lactamase was completely and rapidly inactivated. At a 0.2 μM concentration of olivanate ($[C]_0/[E]_0 = 50$), a k_i value of 0.02 s^{-1} was found. No hydrolysis could be detected.

DISCUSSION

The data presently available in the literature reveal similarities in the modes of action of clavulanic acid and sulbactam (Knowles, 1985). In both cases, there are three possible fates for the initially formed acyl-enzyme (EC*): it can undergo hydrolysis, leading to free enzyme (E), reversible tautomerization to yield a more stable species (EC**), leading to transiently inhibited enzyme, or an irreversible modification into a definitively inactivated enzyme (EC_1*). In the case of sulbactam, this latter complex seemed to be formed by a transamination reaction with an enzyme lysine residue, thus resulting in the cross-linking of two active-site side chains (Brenner & Knowles, 1984). A similar cross-linking reaction has been proposed for clavulanate, but the experimental evidence was less convincing. Formation of the initial acyl-enzyme (EC*) is accompanied by the opening of the C^5–O^1 (clavulanate) or C^5–S^1 (sulbactam) bonds. Whatever the chemical mechanism, kinetically the interactions between these inactivators and several enzymes appeared to be well represented by Model 3, where intramolecular events and a reaction with a second active-site side chain occur in competition with hydrolytic deacylation, so that complete inactivation is only accomplished over a number of turnovers. Model 3 seemed to apply in the interactions between clavulanate and the TEM-2 (Fisher et al., 1978; Charnas et al., 1978; Charnas and Knowles, 1981b; Reading and Farmer, 1981), *Klebsiella pneumoniae* E70 (class A) and *Proteus mirabilis* C889 (undetermined class; Reading and Farmer, 1981) and the *Actinomadura* R39 (Frère et al., 1982b) β-lactamases and between sulbactam and the TEM-2 (Brenner and Knowles, 1981; Kemal and Knowles, 1981; Brenner and Knowles, 1984) and SHV-1 (Labia et al., 1980) β-lactamases. This mechanism might not, however, be universal, since no irreversible inactivation was detected in the reactions between clavulanate and the β-lactamases of *S. aureus* (class A) (Cartwright and Coulson, 1979; Reading and Hepburn, 1979; Rizwi et al., 1989), and *Enterobacter cloacae P99* (class C) (Reading and Farmer, 1981). Also with the *S. aureus* enzyme, no turnover occurs before transient inactivation. The interaction between clavulanate and the *S. cacaoi* β-lactamase seems to offer yet another variation: the value of k_6 is so low that Model 2a better represents the physiologically important phenomenon.

Several other penam sulphones have also been found to be inhibitors of β-lactamases (see, e.g. Cartwright & Coulson, 1979; Fisher et al., 1981; Kelly et al., 1981; Clarke et al., 1983; Knight and Waley, 1985). More recently, Fink and colleagues (1989) observed that inactivation of β-lactamases by penicillin sulphones bearing large hydrophobic side chains was accompanied by a significant change in the protein conformation. They suggested that the inactivation mechanism might depend upon the structure of the C-6 side chain. Actually, it appears that the effectiveness of the sulphone can be modulated through the C-6 and the C-2 substituents (Pratt, 1992).

With the three enzymes studied here, sulbactam seemed to react according to Model 2c, which is also valid for the interaction between β-iodopenicillanate and the *Actinomadura* R39 and *S. albus* G β-lactamases (see below). However, with the latter enzyme, the partition ratio k_3/k_4 was not influenced by ionic strength, in contrast with what was observed with β-halogenopenicillanates (De Meester et al., 1989). This result strengthens the conclusion that the salt effect is a specific characteristic of these latter inactivators (De Meester et al., 1989).

Our results for sulbactam are thus at variance with those of Knowles (1985), who observed an additional branch responsible for a transient inactivation (Model 3). However, the existence of such a branch cannot be ruled out for the enzymes studied here. A rapid equilibrium between initially formed acyl-enzyme and the transient inactivated species would render the latter undetectable and would just lower the apparent K_m and $k_{cat.}$ for hydrolysis. Finally, the $k_{cat.}$ and $(k_i)_{lim}$ observed for the TEM-2 enzyme fall within the range of those measured here. The irreversible inactivation of the *S. albus* G and *S. cacaoi* enzymes was particularly slow.

A thorough kinetic study of the interaction between the TEM-2 β-lactamase and derivatives of olivanic acid has been presented by Knowles and co-workers (Charnas and Knowles, 1981a; Easton and Knowles, 1982). Olivanate MM13902 was found to be a poor substrate of the TEM-2 enzyme (the value of the steady-state turnover rate was 8.5×10^{-4} s^{-1}) and an excellent inactivator (the value of the apparent second-order rate constant for the inactivation process was about 10^6 $M^{-1} \cdot s^{-1}$). The chemical events described for clavulanic acid and sulbactam cannot apply to olivanic acid, for which no rupture of the bond between positions 1 and 5 occurs, yet chemical diversion from the normal course of the catalysed hydrolytic reaction also results in transient enzyme inactivation (Knowles, 1985). The first formed acyl-enzyme contains a δ-2 pyrroline and may either undergo deacylation or tautomerize to a more stable δ-1 pyrroline. This competitive process can be described by Model 2a, where the δ-1 pyrroline may deacylate directly ($k_5 \neq 0$). Alternatively, back tautomerization to the δ-2 pyrroline may occur alone ($k_{-4} \neq 0$, $k_5 = 0$) or in competition with the hydrolytic process (k_{-4} and $k_5 \neq 0$), but, as stated in the Materials and methods section, it is extremely difficult, if not impossible, to distinguish between these three possibilities.

Our results with the *Actinomadura* R39 and *S. albus* G β-lactamases also indicated the occurrence of a branched pathway, but inactivation appeared to be irreversible, in good agreement with Model 2c. Re-activation might have been too slow to be detectable under our experimental conditions. With the *S. cacaoi* enzyme, no hydrolysis could be detected, suggesting a somewhat different model.

Although these compounds have not been studied here, a discussion of β-lactamase inactivators would not be complete if 6β-halogenopenicillanates were not mentioned. Indeed, they have been extremely valuable for labelling and identifying the active serine residue of many enzymes (Knott-Hunziker et al., 1979; Cohen & Pratt, 1980; Joris et al., 1984; Joris et al., 1986; Joris et al., 1987; De Meester et al., 1987b; Amicosante et al., 1988). They are much more efficient as inactivators of the class A than the class C enzymes, thus supplying an easy method for distinguishing between the two classes (De Meester et al., 1986). With β-iodopenicillanate, branched pathways seem to prevail with some enzymes, while no hydrolysis occurs with others (class C) (De Meester et al., 1986). Note that, with the *Actinomadura* R39 enzyme, the published k_3/k_4 value of 80 (Frère et al., 1982a) must be corrected to 150, since the former value was computed on the basis of an erroneous M_r value (15000 as against 29000 deduced from the amino acid sequence).

The prevalence of Models 2c or 2b depended not only on the considered enzyme, but also on the nature of the halogen atom (De Meester et al., 1989). Inactivation was accompanied by the rearrangement of the penam moiety into a dihydrothiazine chromophore and the reaction pathway seemed to involve an

Table 2 Overview of the interaction between inactivators and β-lactamases of classes A and C

The first value in each case refers to the relevant model(s). The first letter refers to the value of $k_{cat.}/K_m$ or k_2/K': H > 100000 $M^{-1} \cdot s^{-1}$ > M > 1000 $M^{-1} \cdot s^{-1}$ > L > 1 $M^{-1} \cdot s^{-1}$. The second letter refers to the rate of re-activation of the transiently inactivated enzyme: 0.1 s^{-1} > S > 10^{-4} s^{-1} > 0. References: 1, Brenner and Knowles, 1981; 2, Brenner and Knowles, 1984; 3, Cartwright and Coulson, 1979; 4, Charnas et al., 1978; 5, Charnas and Knowles, 1981a; 6, Charnas and Knowles, 1981b; 7, Cohen and Pratt, 1980; 8, De Meester et al., 1986; 9, De Meester et al., 1989; 10, Easton and Knowles, 1982; 11, Fisher et al., 1978; 12, Fisher et al., 1981; 13, Frère et al., 1982a; 14, Frère et al., 1982b; 15, Kemal and Knowles, 1981; 16, Knott-Hunziker et al., 1980; 17, Labia et al., 1980; 18, Lenzini and Frère, 1985; 19, Pratt and Cahn, 1988; 20, Reading and Hepburn, 1979; 21, Reading and Farmer, 1981; 22, Rizwi et al., 1989; 23, Yamaguchi et al., 1983.

Compound	*Actinomadura* R39			*S. albus* G			*S. cacaoi*			TEM-1/2			Other class A			Class C			References
Clavulanic acid	3	H	0	3/2c	H	0	3/2a	H	0	3	M	0	3/2a/2'a	H	S/0	2'a	L	0	3, 4, 6, 11, 14, 20, 21, 22
Sulbactam	2c	H	0	2c	H	0	2c	M	0	3	H	0	Irreversible inhibitor*			3	H	0	1, 2, 12, 15, 17, 23
6β-Iodopenicillanic acid	2c	H	0	2c	H	0	2b	M	0	2c	H	0	2b/2c	M	0	2b	L	0	7, 8, 9, 13, 16, 18, 19
Olivanic acid	ND†	M	0	ND	M	0	ND	H	0	2a	H	S	ND	ND	ND	ND	ND	ND	5, 10

* Reported without quantitative details.
† ND, not determined (no data).

intermediate episulphonium, without rupture of the C^5–C^6 bond (Pratt and Cahn, 1988; De Meester et al., 1989).

This overview of the interactions between class A β-lactamases and specific inactivators underlines the diversity of the behaviours of these enzymes, a conclusion which agrees well with their diverse catalytic properties. Indeed, previous studies show that 'classical' β-lactams or 'β-lactamase-stable' compounds can behave very differently if various enzymes are studied (Matagne et al., 1990, 1993a, b).

Table 2 presents a synthesis of the results obtained here and available in the literature (including 6β-iodopenicillanate). It is not always easy to distinguish between schemes which only differ by the presence or absence of one branch (i.e. Model 2a is Model 3 with $k_6 = 0$), and the data in the literature do not always present detailed analyses. The most striking conclusion is that class A β-lactamases behave as a very heterogeneous group in the presence of their mechanism-based inactivators.

The only safe prediction that can be made seems to be that it is nearly impossible to infer the exact reaction pathway of a potential inactivator from the fact that the enzyme belongs to class A. The recent discovery of clavulanate-resistant TEM variants also shows that there might be no such thing as an 'universal' class A inactivator (Thomson & Amyes, 1992).

In this study and in the study of 'β-lactamase-stable' compounds (Matagne et al., 1993a, b), we have not encountered any compound whose inactivating potency only rested on a conformation change at the level of the acyl-enzyme. These destabilizing substrates, termed A-type substrates by Citri and colleagues (1976), have received much attention from Citri (Citri et al., 1976; Klemes and Citri, 1980), Fink (Fink et al., 1987), Pain and Virden (Virden et al., 1978; Pain and Virden, 1979; Carrey et al., 1984; Persaud et al., 1986) and Waley (Kiener and Waley, 1977; Kiener et al., 1980; Thornewell and Waley, 1992) and their collaborators (see also Hardy and Kirsch, 1989). Although such modifications of the enzyme structure can certainly not be ruled out in the various cases encountered here, they do not appear to represent the sole phenomenon involved in the loss of enzyme activity. Interactions with the various compounds could yet result in conformational changes, confined to the active-site or extended to the bulk tertiary structure of the studied enzymes. These conformational constraints could undoubtly contribute to enzyme inactivation and should not be neglected. It is indeed likely that many of these acyl-enzymes, derived from extensive rearrangements (obtained, e.g., with cefoxitin, moxalactam, sulbactam, clavulanic acid or olivanic acid), are inert for non-covalent (conformational) reasons as well as covalent (Pratt, 1989, 1992).

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