RESEARCH COMMUNICATION

Interactions between active-site-serine β-lactamases and compounds bearing a methoxy side chain on the α-face of the β-lactam ring: kinetic and molecular modelling studies

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The interactions between three class A β-lactamases and compounds bearing a methoxy side chain on the α-face of the β-lactam ring (cefoxitin, moxalactam and temocillin) have been studied. When compared with the situation prevailing with good substrates, both acylation and deacylation steps appeared to be severely impaired. Molecular modelling studies of the structures of the Henri–Michaelis complexes and of the acyl-enzymes indicate a major displacement of the crystallographically observed water molecule which connects the glutamate-166 and serine-70 side chains and underline the role of this water molecule in both reaction steps.

INTRODUCTION

Cefoxitin, moxalactam and temocillin (Figure 1) appear to be remarkably resistant to the hydrolytic activity of the active-site-serine β-lactamases. This resistance is thought to be correlated with the presence of the methoxy group on the α-face of the β-lactam ring, attached to C-6 of temocillin and C-7 of cefoxitin and moxalactam. Cefoxitin was found to be a very poor substrate of the TEM-2 [class A: the TEM-1 and TEM-2 β-lactamases differ only by one residue (Q39K) and cannot be distinguished on the basis of their catalytic properties] β-lactamase (Fisher et al., 1980). Hydrolysis proceeds through an acyl-enzyme intermediate with very low acylation and deacylation rate constants (2.4×10^{-2} and 0.48×10^{-2} s^{-1} respectively). The enzyme is thus transiently inactivated as a rather stable acyl-enzyme ($t_{\frac{1}{2}}$ 2.5 min; Fisher et al., 1980). In addition, Citri et al. (1984) provided evidence that this antibiotic induced a conformational change in the enzyme, leading to an increased susceptibility to proteolytic digestion. As suggested by these authors, such an unfavourable conformation would be responsible for transient enzyme inactivation. Faraci and Pratt (1986) underlined the importance of the C-3′ leaving group, whose elimination at the acyl-enzyme stage appeared to stabilize the covalent adduct. Similarly, moxalactam did not interact very efficiently with the TEM-1/2 and *Klebsiella pneumoniae* K1 enzymes (class A) (Labia, 1982; Bush et al., 1982). By contrast, both cefoxitin and moxalactam readily acylate class C β-lactamases with second-order rate constants as high as 4×10^6 $M^{-1} \cdot s^{-1}$ (Galleni et al., 1988) and their resistance to these enzymes rests on slow deacylation steps (0.01–0.3 s^{-1}).

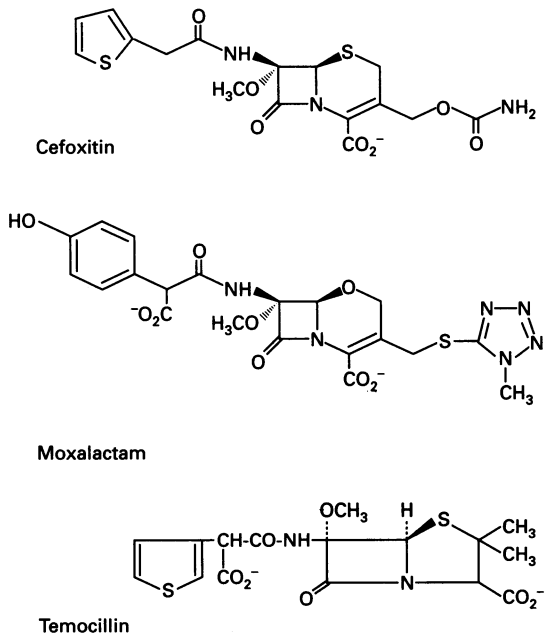

Figure 1 Structures of the studied molecules

Finally, temocillin was not significantly hydrolysed by a wide range of β-lactamases, including class A and class C enzymes (Edmonson et al., 1981; Jules and Neu, 1982; Edmonson and Reading, 1985).

In the present study we analysed the interactions between these compounds and three class A β-lactamases and, on the basis of crystallographic structures and molecular modelling studies, we propose a plausible explanation for the very low acylation and deacylation rates which were observed.

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).

Abbreviation used: PBP, penicillin-binding protein.

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β-Lactam compounds

Cefoxitin, moxalactam and temocillin were kindly given by Merck, Sharp and Dohme Research Laboratories (Rahway, NJ, U.S.A.), Eli Lilly and Co. (Indianapolis, IN, U.S.A.) and Beecham Pharmaceuticals (Brentford, Middx., U.K.). Nitrocefin was purchased from Oxoid (Basingstoke, Hants., U.K.).

Kinetic model

Scheme 1 presents the general model proposed by Faraci and Pratt (1986) for the interaction between active-site-serine β-lactamases and compounds such as cefoxitin and moxalactam, which exhibit a leaving group on C-3′. With temocillin, this reaction cannot occur and $k_4 = 0$ (model 1a). In addition, if k_3 is very low, or equal to zero, temocillin becomes a transient or irreversible inactivator (model 1b), and, similarly, if $k_2 = 0$, it behaves as a competitive inhibitor (model 1c). With cefoxitin and moxalactam, the complete model 2a can sometimes be simplified as follows.

(i) $k_3 = 0$ 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′).

(ii) k_3 and k_5 are negligible: C is an inactivator and the enzyme eventually becomes completely immobilized, generally as the rearranged acyl-enzyme (EC**) (model 2b).

Determination of the kinetic parameters

Progressive inactivation of the enzymes could be monitored discontinuously by measuring the residual activity after increasing periods of time, thus yielding a pseudo-first-order rate constant for inactivation (k_i). However, when possible, enzyme inactivation was monitored continuously, with the help of the reporter substrate method (De Meester et al., 1987). An example of such a determination is given in Figure 2. When inactivation was incomplete, a steady state was eventually reached and the pseudo-first-order rate constant, k_i, was computed as described by De Meester et al. (1987). For all the models described above, with the exception of model 2a, the value of k_i is given by the following equation:

$$k_i = k_r + k_2[C]/(K' \cdot \alpha + [C]) \quad (1)$$

where $k_r = 0$ for models 1b and 2b, k_3 for model 1a and k_5 for model 2a′. The correction factor $\alpha = 1 + [S]/K_{m,S}$ applied when the reporter substrate (S) was utilized. For model 2a, the value of k_i was more complex and of little practical interest.

In each case the k_i values were measured at various C concentrations and when k_i exhibited a hyperbolic variation versus [C], the individual values of k_2 and K' were computed

$$E + C \underset{k_1}{\overset{k_1}{\rightleftharpoons}} EC \xrightarrow{k_2} EC^* \xrightarrow{k_3} E + P$$
$$\qquad\qquad\qquad\qquad\quad \downarrow k_4$$
$$\qquad\qquad\qquad\qquad\quad EC^{**} \xrightarrow{k_5} E + P'$$

Scheme 1 Interaction between active-site-serine β-lactamases and cefoxitin (Faraci and Pratt, 1986)

E is the enzyme, C is cefoxitin and EC* the acyl-enzyme. The k_4 step corresponds to the elimination of the C-3′ leaving group, leading to a second acyl-enzyme, EC**. This complete model is referred to as 'model 2a' in the text. Specific values of some constants lead to the following limit cases: (1) $k_4 = 0$, model 1a; (2) $k_4 = k_3 = 0$, model 1b; (3) $k_2 = 0$, model 1c; $k_3 \ll k_4$, model 2a′; (5) $k_3 = k_5 = 0$, model 2b.

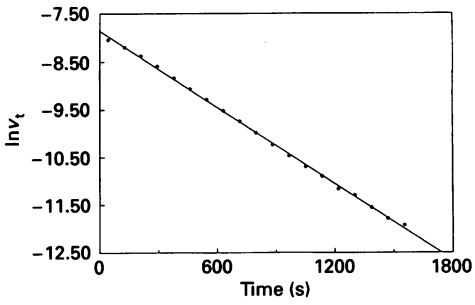

Figure 2 Determination of a k_i value for temocillin and the *Actinomadura* R39 enzyme ($[C]_0 = 4.2$ mM; $[E]_0 = 0.28$ nM)

Nitrocefin (100 μM) was the reporter substrate. Absorbance values (482 nm), representing the averages of 14 individual readings (one reading every 6 s), were used to compute v_0 and v_t (see De Meester et al., 1987) and linearization was performed according to the equation:

$$\ln v_t = \ln v_0 - k_i t$$

yielding a k_i value of 2.7×10^{-3} s^{-1}.

from linearized forms of eqn. (1) or by non-linear regression, using the ENZFITTER program (Leatherbarrow, 1987). When the K' value was too high, only the k_2/K' ratio could be obtained.

When the reporter substrate (S) utilization reached a steady state (v_{ss}), the K_m for the studied compound C ($K_{m,C}$) could also be computed with the help of the following equation:

$$v_0/v_{ss} = 1 + K_{m,S}/(K_{m,S} + [S]) \cdot [C]/K_{m,C} \quad (2)$$

which accounts for the competition between the two substrates at the steady state and 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 buffer, pH 7.0. Dilutions of the enzymes below a concentration of 0.1 mg/ml were performed with buffer solutions containing 0.1 mg/ml of BSA. Hydrolysis of the various compounds was directly recorded at 260 nm (cefoxitin and moxalactam) or 235 nm (temocillin).

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

The concentration of nitrocefin (100 μM) was well below the K_m values for the *S. albus* G (1100 ± 140 μM) and *S. cacaoi* (1300 ± 120 μM) enzymes, so that the correction factor, α, was negligible in these cases. For the β-lactamase of *Actinomadura* R39 ($K_m = 70 \pm 5$ μM), α was 2.4. In consequence, no large errors were introduced (De Meester et al., 1987).

Absorbance measurements were performed with the help of microcomputer-linked Beckman DU-8 and 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). The structure of the adduct formed upon acylation of the *Bacillus*

Table 1 Kinetic parameters for the interactions between the three enzymes and cefoxitin, moxalactam and temocillin

The capital letter in parentheses refers to the method utilized for obtaining the value: (A), reporter substrate; (B), competitive inhibition in short (< 60 s) experiments; (C), direct hydrolysis.

Compound	Parameter	Species... *Actinomadura* R39	*S. albus* C	*S. cacaoi*
Cefoxitin	k_2 (s^{-1})	$(1.35 \pm 0.08) \times 10^{-3}$ (A)	$< 2 \times 10^{-4}$ (A)	0.1 ± 0.01 (A)
	K' (mM)	0.85 ± 0.08 (A–B)	8 ± 2 (B)	2 ± 0.2 (A)
	k_2/K' ($M^{-1} \cdot s^{-1}$)	1.65 ± 0.1 (A)	–	48 ± 4 (A)
	k_3 or k_5 (s^{-1})	$< 2 \times 10^{-5}$ (A–C)	–	$(6 \pm 2) \times 10^{-4}$ (A)
	Conclusion...	Poor inactivator	Poor competitive inhibitor	Poor inactivator/very poor substrate
	Model...	1b or 2b	1c	1a or 2a′
Moxalactam	k_2 (s^{-1})	$> 2 \times 10^{-3}$ (A)	–	
	K' (mM)	> 10 (A)	> 1 (A)	Complex interaction (see text)
	k_2/K' ($M^{-1} \cdot s^{-1}$)	0.35 ± 0.1 (A)	< 0.03 (A)	
	k_3 or k_5 (s^{-1})	$< 2 \times 10^{-4}$ (A)	–	$(k_{cat.})_{SS} = 7 \times 10^{-3}$ s^{-1}
	Conclusion...	Poor inactivator	No interaction	Branched pathway: very poor substrate
	Model...	1b or 2b	–	2a
Temocillin	k_2 (s^{-1})	0.01 ± 0.004 (A)	< 0.001 (A)	$< 2 \times 10^{-5}$ (A)
	K' (mM)	5 ± 2 (A)	15 ± 5 (B)	0.18 ± 0.02 (B)
	k_2/K' ($M^{-1} \cdot s^{-1}$)	2 ± 0.5 (A)	< 0.1 (A)	< 0.1 (A)
	k_3 (s^{-1})	$< 2 \times 10^{-4}$ (A–C)	–	–
	Conclusion...	Poor inactivator	Very poor competitive inhibitor	Competitive inhibitor
	Model...	1b	1c	1c

licheniformis β-lactamase by cefoxitin was similarly modelled on the basis of the available structure (Moews et al., 1990; Knox and Moews, 1991; Protein Data Bank Entry 4BLM).

RESULTS

Kinetic results

The results are summarized in Table 1. With the exception of the interaction between moxalactam and the β-lactamase from *S. cacaoi*, they could be interpreted on the basis of models of type 1. However, the presence of the leaving group on C-3′ of these compounds suggests that the accumulated acyl-enzyme was probably the rearranged adduct, EC**.

When moxalactam was used at concentrations above 100 μM as a substrate of the *S. cacaoi* enzyme, a burst was observed, followed by a steady state. The size of the burst was larger than the enzyme concentration, and the half-transition time was 11 min. The rates at zero time (v_0) and at the steady state (v_{ss}) were respectively 0.03 ± 0.003 and 0.014 ± 0.002 μmol·min^{-1}·mg^{-1}. A K_i value of 25 ± 2 μM was obtained in short (< 60 s) competition experiments with nitrocefin. It could thus be concluded that $(k_{cat.})_0$ and $(k_{cat.})_{SS}$ were 0.016 and 0.007 s^{-1} respectively and that model 2a applied.

Modelling of the interactions with the *S. albus* G β-lactamase

The structure of the active site of the *S. albus* G β-lactamase has been minimized, and the docking of two good substrates (benzylpenicillin and cephalosporin C) into this active site has been analysed in detail (Lamotte-Brasseur et al., 1991). Ticarcillin only differs from temocillin by the absence of the methoxy group on C-6. Figures 3(a) and 3(b) compare the minimized structures of the Henri–Michaelis complexes formed when both compounds are docked in the *S. albus* G enzyme active site and demonstrate that the major difference is the position of the water molecule W1. In the free enzyme, this water molecule forms a connecting bridge between the side chains of Ser^{70} and Glu^{166}. As observed previously with benzylpenicillin and cephalosporin C, the binding of ticarcillin barely modifies the position of W1. By contrast, the methoxy group of temocillin completely displaces the water molecule. A similar displacement of W1 was observed when cefoxitin and moxalactam were docked in the enzyme active site (results not shown).

Since the *S. albus* G enzyme was not acylated by any of the three compounds containing a methoxy group, it did not seem useful to perform the modelling of corresponding acyl-enzymes with this β-lactamase. However, the three-dimensional structure of the homologous *B. licheniformis* β-lactamase is available (Moews et al., 1990; Knox and Moews, 1991). This class A enzyme is slowly acylated by cefoxitin and deacylation is even slower ($t_{\frac{1}{2}} = 75 \pm 10$ min; M. Jamin, C. Damblon, A. M. Misselyn-Bauduin and J. M. Frère, unpublished work). Modelling of the corresponding acyl-enzyme (Figure 3c) shows a displacement of the water molecule, which is expected to act as a nucleophile in the deacylation reaction. This motion is similar to that observed in the Henri–Michaelis complexes described above in the interactions between the *S. albus* G β-lactamase and the three compounds containing the α-methoxy group.

DISCUSSION

Temocillin appears to interact with the three enzymes according to a linear pathway. There is no significant hydrolysis and the compound behaves as a poor competitive inhibitor (*S. albus* G) or a poor inactivator (*Actinomadura* R39). Only with the *S. cacaoi* enzyme is better binding observed, but very little acylation occurs anyway. With the two other compounds, evidence for a branched pathway (model 2a) could only be found for the moxalactam–*S. cacaoi* lactamase interaction. The other interactions could be explained on the basis of linear pathways, and the results of Faraci and Pratt (1986) indicate that the acyl-enzyme, when formed, probably rearranges with elimination of the C-3′ leaving group more rapidly than the first acyl-enzyme

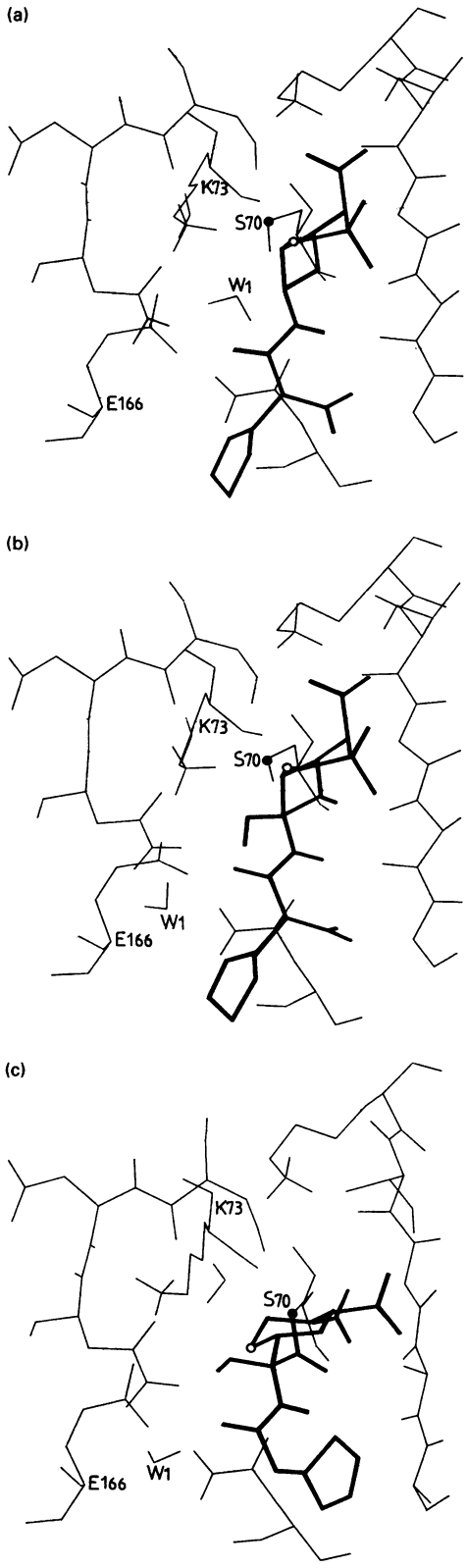

Figure 3 Modelling of two Henri–Michaelis complexes (a and b) and of an acyl-enzyme (c)

The hydroxy oxygen of the active Ser^{70} and the S-1 atoms of the antibiotics are shown as closed and open circles respectively. The antibiotic structure is represented by **bold** lines. (**a**) and (**b**) Docking of ticarcillin (**a**) and temocillin (**b**) in the active site of the *S. albus* G β-lactamase. With ticarcillin, the k_2 and k_3 values are larger than 100 s^{-1}. (**c**) Modelled structure of the acyl-enzyme formed upon interaction of cefoxitin with the *B. licheniformis* β-lactamase. The structure has been minimized with the rearranged cefoxitin moiety, where the C-3′ leaving group has been eliminated (EC** in Scheme 1). Similar modelling performed on the initial EC* adduct did not indicate a different positioning for the two C-7 side chains, although the orientation of the six-membered dihydrothiazine ring was understandably modified.

can undergo hydrolysis. The *S. cacaoi* enzyme was the only one with which some hydrolysis of moxalactam and cefoxitin was observed, but $k_{cat.}$ values remained extremely low ($< 10^{-2}$ s^{-1}). Despite the slight differences illustrated in Table 1, and in agreement with all the available data, a rather clear pattern emerges for the behaviour of class A β-lactamases and compounds containing a methoxy group on the α-face of the β-lactam ring: acylation, when it occurs, is extremely slow, and deacylation is even slower, both several orders of magnitude below the rates observed with good substrates.

The molecular modelling studies presented here supply a tentative explanation for these observations. The water molecule, W1, which was hypothesized to act as an intermediate in the activation of the Ser^{70} hydroxy group by Glu^{166} acting as a general base (Lamotte-Brasseur et al., 1991) is displaced by the α-methoxy group of the substrate, making acylation very unlikely. These studies were performed with the *S. albus* G β-lactamase, but it is important to realize that a water molecule was found in exactly the same position, between the Ser^{70} and Glu^{166} side chains, in all class A β-lactamases whose three-dimensional structures have been determined (Herzberg, 1991; Knox and Moews, 1991; Strynadka et al., 1992).

Thus our modelling results can probably be safely extended to most, if not all, interactions between class A β-lactamases and compounds containing an α-methoxy group in that strategic position. The methoxy group of cefoxitin also appears to induce a similar displacement of the water molecule at the level of the acyl-enzyme, thus explaining the very low deacylation rates.

Strynadka et al. (1992) have proposed the deprotonated Lys^{73} ϵ-NH_2 as the general base responsible for the activation of the Ser^{70} hydroxy group. However, Figure 3 shows that the relative positions of the Lys^{73} and Ser^{70} side chains are nearly superimposable in the Henri–Michaelis complexes formed with temocillin and ticarcillin. The mechanism of Strynadka et al. (1992) thus completely fails to explain the greatly decreased acylation rates observed with the first compound and the wild-type enzyme.

Together with other published data, our results underline a major difference between class A and class C β-lactamases. As already noted in the Introduction, cefoxitin and moxalactam acylate the latter enzymes with high efficiency. This suggests major differences between the acylation mechanisms of class A and class C enzymes. Consequently, it might not be surprising that the search for an acidic residue in class C enzymes, equivalent to the class A Glu^{166}, has remained unsuccessful. The class C Tyr^{150} phenol group has been hypothesized to exhibit a strongly decreased pK_a and to act as a general base (Oefner et al., 1990), but this remains to be confirmed. The position of this residue in the three-dimensional structure is quite different from that of Glu^{166} in the class A enzymes and it originates from a totally different part of the polypeptide chain. It is also striking that cefoxitin, moxalactam and temocillin often acylate penicillin-binding proteins (PBPs) with efficiencies rather similar to those of related β-lactam compounds devoid of the α-methoxy side chain (e.g. the *Streptomyces* K15 and R61 DD-peptidases; Frère and Joris, 1985; Leyh-Bouille et al., 1986), and one could thus be tempted to conclude that their acylation pathways are more closely related to that of the class C β-lactamases. To our knowledge, situations where acylation is orders of magnitude more efficient than deacylation do not occur with class A β-

lactamases. Indeed, all class A inactivators (e.g. clavulanate, sulbactam and β-iodopenicillanate) owe their efficiency to a very rapid rearrangement of the acyl-enzyme (Knowles, 1985; De Meester et al., 1989). By contrast, with many β-lactams and class C enzymes, acylation is much more efficient than deacylation (Galleni and Frère, 1988; Galleni et al., 1988; Monnaie et al., 1992) and the same is generally true for PBPs (Frère and Joris, 1985). In consequence, it might be reasonable to assume that, with the class A enzymes, acylation and deacylation occur according to 'symmetrical' mechanisms, where the water molecule, W1, would play an important role in both reaction steps. Such a symmetry would not be the rule for class C β-lactamases and PBPs, at least in their interactions with β-lactams. It should,, however, be noted that some modified class A enzymes, obtained by site-directed mutagenesis, appear to exhibit a selectively impaired deacylation step (Escobar et al., 1991). These results remain presently unexplained on the basis of our hypothesis.

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