Biochem. J. (1991) 279, 111–114 (Printed in Great Britain)

Interaction of β-lactamases I and II from *Bacillus cereus* with semisynthetic cephamycins

Kinetic studies

Javier MARTIN VILLACORTA,* Paloma ARRIAGA,† José LAYNEZ† and Margarita MENENDEZ†‡
*Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria, Universidad de León, Campus de Vegazana s/n, 24071 León, and †Instituto de Química-Física 'Rocasolano', Serrano 119, 28006 Madrid, Spain

The influence of C-6α- or C-7α-methoxylation of the β-lactam ring in the catalytic action of class A and B β-lactamases has been investigated. For this purpose the kinetic behaviour of β-lactamases I (class A) and II (class B) from *Bacillus cereus* was analysed by using several cephamycins, moxalactam, temocillin and related antibiotics. These compounds behaved as poor substrates for β-lactamase II, with high K_m values and very low catalytic efficiencies. In the case of β-lactamase I, the substitution of a methoxy group for a H atom at C-7α or C-6α decreased the affinity of the substrates for the enzyme. Furthermore, the acylation of cephamycins was completely blocked, whereas that of penicillins was slowed down by a factor of 10^4–10^5, acylation being the rate-determining step of the process.

INTRODUCTION

Cephamycins are β-lactam antibiotics with a molecular structure similar to that of cephalosporin, but including a methoxy group at position C-7α (Nagarajan *et al.*, 1971). These compounds are much less sensitive to β-lactamase attack than are cephalosporins suggesting an important role of the methoxy group in the mechanism of resistance against β-lactamase hydrolysis, associated with the formation of stable acyl-enzymes whose hydrolysis is rate-determining with regard to turnover of class A and C β-lactamases (Faraci & Pratt, 1986; Lenzini & Frère, 1985; Galleni *et al.*, 1988; Mazella & Pratt, 1989).

The kinetic parameters and mechanism of interaction between β-lactamases and β-lactam compounds has been widely studied in recent years and many points have been clarified concerning their resistance to β-lactam antibiotics. However, many questions remain unanswered (Hou & Poole, 1973; Frère *et al.*, 1988). For this reason, it would seem of great interest to ascertain the molecular basis of the interaction mechanism in order to find broad-spectrum β-lactam antibiotics with high antibacterial activity and resistance to β-lactamase attack.

Here we present kinetic data concerning the interactions between β-lactamase I (class A) and II (class B) from *Bacillus cereus* and several 6α- or 7α-methoxy-β-lactams and related antibiotics. The effect of the methoxy substituent in the interaction with the β-lactamases is discussed.

MATERIALS AND METHODS

β-Lactam compounds

Cefoxitin and cefmetazol were obtained from Merck Sharp and Dohme Research Laboratories (Rahway, NJ, U.S.A.); moxalactam and cephalothin from Eli Lilly and Co. (Indianapolis, IN, U.S.A.); ticarcillin and temocillin from Beecham Research Laboratories (Brentford, Middx., U.K.); cefotetan was from ICI–Farma (Madrid, Spain); nitrocefin was from Glaxo Group Research (Greenford, Middx., U.K.); cefminox was from Meiji Seika Kaisha Research Laboratories (Japan) and SQ 14.359 from Squibb and Sons (Princeton, NJ, U.S.A.). The structures of these antibiotics are shown in Fig. 1.

Enzymes

β-Lactamases I and II from *Bacillus cereus* (569/H/9) were supplied by Porton Products Ltd., Salisbury, Wilts., U.K. The enzyme concentrations were measured spectrophotometrically on the basis of the reported molar absorption coefficients (Arnold & Viswanatha, 1983; Cartwright & Waley, 1987).

Assays

β-Lactamase I and II activities were routinely measured using nitrocefin as substrate (O'Callaghan *et al.*, 1972). The experiments with β-lactamase I were performed in 60 mM-potassium phosphate buffer, pH 7.0. β-Lactamase II assays were carried out in 30 mM-potassium citrate/1 mM-$ZnSO_4$ buffer, pH 7.0. The enzymic hydrolysis of the antibiotics was monitored from the decrease in absorbance resulting from opening of the β-lactam ring, using a Cary 210 spectrophotometer equipped with thermostatically controlled cells (temperature 25±0.1 °C). Cells with 0.1–1 cm pathlength were employed, depending on the solution absorbance. The reaction was monitored at the selected wavelength (between 220 and 350 nm) for which the variation of the absorbance was largest (see Table 1 below), unless the absorption of the substrate was very high. The absorption-coefficient changes, $\Delta\epsilon$, were measured using a large excess of β-lactamase II.

Determination of K_m and $k_{cat.}$

The values of K_m and $k_{cat.}$ for cephalothin and nitrocefin were measured under initial-rate conditions using Lineweaver–Burk and Hanes–Wolf plots. With ticarcillin, a very good substrate for both β-lactamases, K_m and $k_{cat.}$ were measured by analysing the complete progress curves of the reaction (Wharton & Szawelski, 1982).

For the different cephamycins, temocillin and moxalactam, the K_m values were determined as K_i values. The catalytic constants of these antibiotics were calculated from the complete curves of degradation under first-order kinetic conditions ([S] $\ll K_m$). The kinetic constants of cefoxitin, cefotetan and cefminox with β-

‡ To whom correspondence should be addressed.

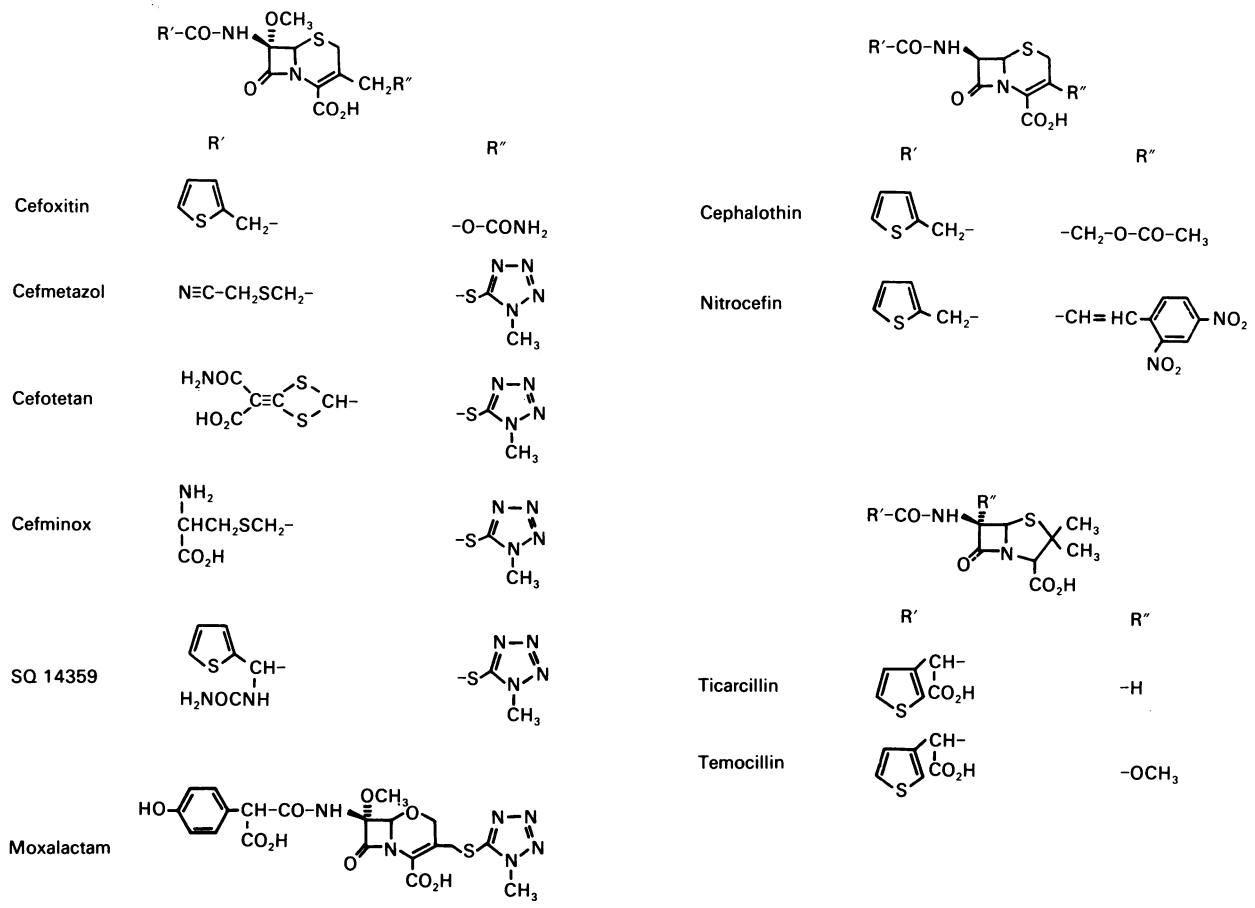

Fig. 1. Structures of the substrate molecules studied

lactamase II were also determined under initial-rate conditions, but in these cases the reactions were performed outside the spectrophotometer due to the large concentrations of substrate employed (1–27 mM). The absorbance changes were monitored by appropriate dilution of portions of the reaction mixtures into a cuvette with buffer at different reaction times. In these three cases both K_m and K_i values were employed to calculate $k_{cat.}$ from the experiments under first-order conditions.

Determination of K_i

The K_i values for β-lactamases I and II for the different cephamycins, temocillin and moxalactam, were determined from the secondary Lineweaver–Burk plot, by using nitrocefin as substrate.

Inactivation experiments

β-Lactamases I and II (1–10 μM) were incubated with the various antibiotics (5–15 mM) for 0–115 min. At different times, aliquots were removed from the incubation mixtures, diluted 500-fold in the appropriate buffer and then the activity assayed using nitrocefin as above. Control experiments (no antibiotic in the incubation mixture) were run simultaneously under the same conditions.

RESULTS

The inhibition studies showed that the 7α- and 6α-methoxy-β-lactams included in the present study behave as very poor competitive inhibitors of β-lactamases I and II, as can be seen from the K_i constants listed in Tables 1 and 2. Significantly, the inhibition constants of β-lactamase I are one order of magnitude higher than those of β-lactamase II, with the exceptions of cefoxitin and temocillin, the only 6α-methoxy penicillin derivative included.

On incubation with β-lactamase II, the antibiotics yielded difference spectra with minima around 270–280 nm (7α-methoxy derivatives) and 236 nm (temocillin), indicating the opening of the β-lactam ring. The kinetic constants measured from the antibiotic hydrolysis are reported in Table 1. Temocillin was the only antibiotic of this series whose interaction with β-lactamase I resulted in an extremely slow degradation, for which a $k_{cat.}$ value of 0.06 s^{-1} was estimated. The results obtained for the incubation of either β-lactamase I or II with these antibiotics and the subsequent dilution with nitrocefin allow us to rule out a time-dependent formation of inactivated states, since the initial activities in all cases were the same as those obtained in the blank experiments run in the absence of inhibitor, regardless of the incubation time (0–115 min).

In order to gain further information about the influence of the methoxy group in position C-6α or C-7α, other compounds containing the β-lactam ring were included in the present study. The kinetic parameters of β-lactamases II and I for these antibiotics are listed in Tables 1 and 2 respectively.

DISCUSSION

The values of $k_{cat.}$ (0.07–1 s^{-1}) and $k_{cat.}/K_m$ (33–143 $s^{-1} \cdot M^{-1}$) listed in Table 1 show that methoxy derivatives are poor substrates of β-lactamase II, suggesting that the presence of this group hampers the recognition of the substrate by the enzyme.

Table 1. Kinetic parameters of *Bacillus cereus* β-lactamase II for 7α- and 6α-methoxy-β-lactams, cephalothin, ticarcillin and nitrocefin

Antibiotic	$\Delta\epsilon$ ($M^{-1}\cdot cm^{-1}$)	λ (nm)*	K_i (mM)	K_m (mM)	$k_{cat.}$ (s^{-1})	$k_{cat.}/K_m$ ($s^{-1}\cdot M^{-1}$)
Cefoxitin	5377	265	1.9±0.3	3.8±0.7	0.3±0.1§ 0.26±0.03‖	79
Cefotetan	5386	260	1.9±0.3	2.8±1	0.13±0.03§ 0.16±0.03‖	46
Cefminox	4436	272	3.2±0.4	3±1	0.2±0.1§ 0.21±0.03‖	63
Cefmetazol	10965	274	7±1	–	0.09±0.01§	13
SQ 14359	3360	275	1.7±0.3	–	0.07±0.01§	41
Moxalactam	9700†	270	7±1	–	1.00±0.01§	143
Temocillin	510	236	15±3	–	0.5±0.1§	33
Cephalothin	7392	260	–	$(52\pm9)\times10^{-3}$	132±9	2538×10^3
Ticarcillin	639	236	–	0.2±0.03	207±18	1035×10^3
Nitrocefin	15900‡	482	–	$(5.2\pm0.5)\times10^{-3}$	11±1	2111×10^3

* Wavelength at which $\Delta\epsilon$ was measured.
† Kobayashi *et al.* (1986).
‡ O'Callaghan *et al.* (1972).
§ Under first-order kinetic conditions.
‖ From initial-rate measurements.

Table 2. Kinetic parameters of *Bacillus cereus* β-lactamase I for 7α- and 6α-methoxy-β-lactams, cephalothin, ticarcillin and nitrocefin

Antibiotic	K_i (mM)	K_m (μM)	$k_{cat.}$ (s^{-1})	$k_{cat.}/K_m$ ($s^{-1}\cdot M^{-1}$)
Cefoxitin	7±2	–	–	–
Cefotetan	≃36	–	–	–
Cefminox	≃92	–	–	–
Cefmetazol	≃92	–	–	–
Moxalactam	*	–	–	–
Temocillin	14±1	–	0.06±0.02†	4
Cephalothin	–	80±9	0.7±0.03	8×10^3
Ticarcillin	–	200±100	620±25	3100×10^3
Nitrocefin	–	24±2	20±1	833×10^3

* No inhibition is detected.
† Under first-order kinetic conditions.

The effects linked to the side-chain modifications are in general slight, especially when considering that the $k_{cat.}$ values and the catalytic efficiencies were remarkably low for all the antibiotics tested. In general, 7α-methoxycephalosporins have higher K_m values for β-lactamase I than for Zn^{2+}-dependent enzymes, whereas temocillin has a similar value for both. Incubation of cephamycins with β-lactamase I resulted in no degradation of the antibiotics and the incubation–dilution experiments showed that the recovery of activity was instantaneous in the presence of a good substrate like nitrocefin. These findings show that β-lactamase I binds to these antibiotics in the form of a reversible Michaelis–Menten complex. Thus, substitution of a methoxy group for an H atom at C-7α blocks their degradation by β-lactamase I by preventing the formation of the acyl enzyme. Therefore the cephamycin K_i values reported in Table 2 coincide with K_s, the equilibrium constant for the Michaelis–Menten complex:

$$E+S \underset{K_s}{\rightleftharpoons} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E+P$$

Scheme 1

The catalytic constant estimate for temocillin under first-order conditions indicates that acylation is also the rate-determining step, since no lag is observed after incubation of this antibiotic with β-lactamase I. The insertion of the methoxy group into the C-6α position of the ring skeleton of penicillin causes the second-rate-order constant to decrease by a factor of 10^6, as can be seen from the catalytic efficiencies for temocillin and ticarcillin (see Table 2).

We shall now analyse the influence of the methoxy substituent on the catalytic behaviour of both classes of β-lactamases by comparing the kinetic parameters for the antibiotic pairs cefoxitin/cephalothin and temocillin/ticarcillin (see Fig. 1). Ticarcillin is a good substrate for β-lactamase I, for which both the rate constants for acylation and deacylation should be high (see Table 2) and could contribute to the catalytic constant, as is the case with other substrates (Martin & Waley, 1988). The catalytic constants of β-lactamase I for cephalothin might indicate deacylation as the rate-determining step for the hydrolysis of this antibiotic. Nevertheless, at the present level of information no definite conclusions can be drawn. Thus, C-6α or C-7α methoxylation would increase the dissociation constant, K_s, by a factor of about 10–10^2, depending on whether deacylation ($K_s = K_m\cdot k_2/k_3 \geqslant 10\,K_m$, since $k_3 \leqslant 10\,k_2$) or acylation ($K_m = K_s$) were the rate-determining step for the hydrolysis of ticarcillin and cephalothin. Analogously, the methoxy group at C-6α could make the acylation process between 10^5 (if $k_{cat.} \simeq k_3$) and 10^4 (if $k_{cat.} \simeq k_2$) times slower. The estimated effect on K_s is in the order of the value reported for the TEM 2 β-lactamase of *Escherichia coli*, using the antibiotic pair cephalothin/cefoxitin (Faraci & Pratt, 1986). From the present results it can be concluded that the resistance of 7α- and 6α-methoxy-β-lactams to the action of β-lactamase I is determined by their low affinity for the enzyme (high K_s values) and by acylation being blocked by this substituent. This behaviour distinguishes β-lactamase I from other enzymes belonging to class A, such as the enzymes from *Streptomyces cacaoi* and TEM-2 from *E. coli*, when using substrates with good leaving groups at C-3, such as cefoxitin. In these cases, deacylation of the covalent complex after elimination of the C-3 substituent was the rate-determining step in the hydrolysis of cephamycins, despite the fact that the major effect of methoxylation was also observed in the acylation rate (Lenzini & Frère, 1985; Faraci & Pratt, 1986). A similar behaviour has been reported for the class C β-lactamases so far studied (Galleni *et al.*, 1988; Mazella & Pratt, 1989). In this way, the behaviour of the *B. cereus* enzyme seems more similar to that of the β-lactamase from *Streptomyces albus* G (Kelly *et al.*, 1981).

The effect of methoxylation on the catalytic action of β-

lactamase II has been analysed in terms of the steady-state catalytic constants, K_m and $k_{cat.}$, owing to the complex relationships between them and the constants for the elementary steps of the branched kinetic pathway proposed for this enzyme (Bicknell & Waley, 1985; Bicknell *et al.*, 1986). The K_m values of cephalothin or ticarcillin for β-lactamase II are between 40- and 75-fold better than those of cefoxitin or temocillin. These values are similar to those obtained for β-lactamase I. The catalytic constant is even more sensitive to the presence of the methoxy group, and the intermediates formed with cefoxitin or temocillin are between 400 and 500 times more inert than those with the non-substituted antibiotics. In view of the above reported results we can conclude that, regardless of the side-chain modifications, the insertion of the $-OCH_3$ substituent into the C-6α or C-7α position of the β-lactam ring is the main characteristic which determines the affinity for the enzyme and the catalytic efficiency of β-lactamase II with these antibiotics.

We thank Ms. L. Rodriguez Monge for her excellent technical assistance and Mr. G. Keitch for linguistic revision. P.A. acknowledges a fellowship from the Spanish 'Fondo de Investigaciones Sanitarias'. This work was supported by Grant PB87-0352 from the Spanish Comisión Interministerial de Ciencia y Tecnología.

Received 5 February 1991/11 April 1991; accepted 17 April 1991

REFERENCES

Arnold, L. D. & Viswanatha, T. (1983) Biochim. Biophys. Acta **759**, 192–197
Bicknell, R. & Waley, S. G. (1985) Biochemistry **24**, 6876–6887
Bicknell, R., Schäffer, A., Waley, S. G. & Auld, D. S. (1986) Biochemistry **25**, 7208–7215
Cartwright, S. J. & Waley, G. G. (1987) Biochemistry **26**, 5329–5337
Faraci, W. F. & Pratt, R. F. (1986) Biochemistry **25**, 2934–2941
Frère, J.-M., Joris, B., Varetto, L. & Crine, J. M. (1988) Biochem. Pharmacol. **37**, 125–132
Galleni, M., Amicosante, G. & Frère, J.-M. (1988) Biochem. J. **255**, 123–129
Hou, J. P. & Poole, J. W. (1973) J. Pharm. Sci. **62**, 783–788
Kelly, J. A., Frère, J.-M., Duez, C. & Ghuysen, J.-M. (1981) Biochem. J. (1981) **199**, 575–580
Kobayashi, S., Arai, S., Hayashi, S. & Fujimoto, K. (1986) Antimicrob. Agents Chemother. **30**, 713–718
Lenzini, M. V. & Frère, J.-M. (1985) J. Enzyme Inhib. **1**, 25–34
Martin, M. T. & Waley, S. G. (1988) Biochem. J. **254**, 923–925
Mazzela, L. J. & Pratt, R. F. (1989) Biochem. J. **259**, 255–260
Nagarajan, R., Boeck, L. D. & Golman, M. (1971) J. Am. Chem. Soc. **93**, 2308–2310
O'Callaghan, C. H., Morris, A., Kirby, S. M. & Shingler, A. H. (1972) Antimicrob. Agents Chemother. **2**, 442–448
Wharton, CH. W. & Szawelski, R. J. (1982) Biochem. J. **203**, 351–360