

# Interaction of clavulanate with class C $\beta$ -lactamases

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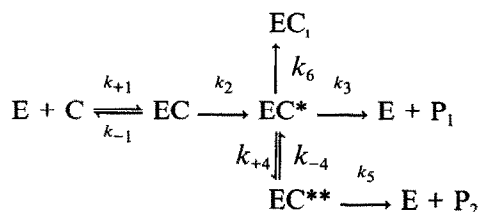
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The interactions between clavulanate and three class C enzymes have been studied in detail. In all cases, the reactions followed branched pathways where 25–150 turnovers occurred before inactivation was completed. Reactivation rates were quite low. The poor efficiency of clavulanate as a class C inactivator appeared to rest upon a very slow acylation of the protein, and of a relatively high turnover rate.

$\beta$ -Lactamase; Clavulanic acid; AmpC; *Enterobacter cloacae* 908R; *Serratia marcescens*; *Escherichia coli* K12

## 1. INTRODUCTION

Class C  $\beta$ -lactamases are quite resistant to clavulanic acid, a  $\beta$ -lactam produced by *Streptomyces clavuligerus*, and which behaves as a very efficient inactivator of class A  $\beta$ -lactamases. This difference was utilised by Bush [1] as a classification criterion for defining group I "cephalosporin-hydrolysing enzymes not inhibited by clavulanic acid". In fact, the sequences of those of the group I enzymes which are well identified show that they belong to class C. The basis of the relative resistance of these enzymes to clavulanate remains however undetermined. The only quantitative data obtained by Reading and Farmer [2] indicated a very low second-order rate constant of  $4 \text{ M}^{-1} \cdot \text{s}^{-1}$  for the inactivation of the *Enterobacter cloacae* P99  $\beta$ -lactamase by clavulanate. Scheme 1 depicts the branched pathway model proposed to explain the interactions between clavulanate and several class A enzymes.



Scheme 1

Where E is the enzyme, C clavulanate, EC\* the acyl-enzyme, EC\*\* and EC<sub>1</sub> rearranged acylenzymes. The  $k_6$  branch is not always present.

Generally,  $k_3$  was much larger than  $k_{+4}$  and  $k_6$ , a steady-state was rapidly established in the horizontal branch, and initial rate experiments, performed before

EC\*\* and EC<sub>1</sub> significantly accumulate, allowed the measurement of the usual parameters  $k_{\text{cat}}$  and  $K_m$  [3]. Similarly in experiments performed at high  $C_0/E_0$  ratios much larger than  $k_3/(k_{+4} + k_6)$  (where  $C_0$  and  $E_0$  are the initial clavulanate and enzyme concentrations), an inactivation rate constant ( $k_i$ ) was determined and

$$k_i = \frac{(k_i)_{\text{lim}} C_0}{K_m + C_0}$$

where  $(k_i)_{\text{lim}}$  characterises the inactivation rate at saturating clavulanate concentrations and is equal to  $k_2(k_{+4} + k_6)/(k_2 + k_3 + k_{+4} + k_6)$  if  $k_{-4}$  and  $k_5$  can be neglected [3].

On this basis, a poor inactivation might result from a low acylation rate (low  $k_2/K'$  where  $K' = (k_{-1} + k_2)/k_1$ ) or from a high  $k_3/(k_{+4} + k_6)$  ratio since

$$k_{\text{cat}} = \frac{(k_3 + k_{+4} + k_6)}{(k_{+4} + k_6)} \cdot (k_i)_{\text{lim}}$$

An additional possibility might be high values of  $k_5$  or  $k_{-4}$ , with  $k_6 = 0$ , but Reading and Farmer also showed that reactivation of the enzyme was quite slow  $k_{\text{reac}} = k_{-4} + k_5 = 0.6 \times 10^{-4} \text{ s}^{-1}$  and that no irreversibly inactivated enzyme accumulated ( $k_6 \sim 0$ ). Thus, the second order rate constant of  $4 \text{ M}^{-1} \cdot \text{s}^{-1}$  corresponded to  $(k_i)_{\text{lim}}/K_m$ . In this paper the interactions between three class C  $\beta$ -lactamases and clavulanate were studied and low acylation rates were found to be responsible for the poor efficiency of the inactivation process.

## 2. MATERIALS AND METHODS

### 2.1. Enzymes

The enzymes were those from *Enterobacter cloacae* 908R, *Serratia marcescens* SC8247 and *Escherichia coli* K12 described in previous studies [4–6]. The enzyme activities were routinely determined using benzylpenicillin or cefazolin. All experiments were performed in 50 mM sodium phosphate buffer, pH 7.0, containing 0.2 M NaCl.

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## 2.2. Steady state experiments

Clavulanate hydrolysis was monitored by following the appearance of an unstable chromophoric group with a extinction coefficient  $\epsilon_{280\text{ nm}} = 2,000\text{ M}^{-1} \cdot \text{cm}^{-1}$ . Because of the instability of this chromophore initial rates of clavulanate turnover were monitored for short times (ca. 60 s) and initial rate values were computed by extrapolating the curve  $[P]/t$  vs.  $[P]$  to  $[P] = 0$  with the help of a simple quadratic equation:  $y = ax^2 + bx + c$ .

## 2.3. Inactivation experiments

The course of enzyme inactivation was studied with  $100\text{ }\mu\text{M}$  cefazolin as a reporter substrate: a solution ( $440\text{ }\mu\text{l}$ ) containing cefazolin and clavulanate was added with  $5\text{ }\mu\text{l}$  of enzyme at various concentrations (the enzyme concentrations were increased at higher clavulanate concentrations). This allowed the determination of  $(k_i)_{\text{lim}}/K_m$  ratios.

## 2.4. Partial inactivation experiments

The enzymes were incubated at different  $C_0/E_0$  ratios such that partial inactivation was observed. After 60 min the samples were diluted 100-fold in buffer, and residual activity was assayed on  $1\text{ mM}$  benzylpenicillin. The remaining activity ( $A$ ) is related to the  $[k_3/(k_{+4} + k_6)]$  ratio and the initial activity  $A_0$  by:

$$\frac{C_0}{E_0} = \frac{k_3}{(k_{+4} + k_6)} \frac{A_0 - A}{A_0}$$

valid when  $k_3 \gg (k_{+4} + k_6)$ . It was verified that no further inactivation occurred upon prolonged incubation.

## 2.5. Reactivation experiments

After incubation in the presence of clavulanate at concentrations of clavulanate allowing complete inactivation within one hour, the enzyme was diluted in buffer and kept at  $30^\circ\text{C}$ . Aliquots were further diluted in  $1\text{ mM}$  benzylpenicillin to assay for recovery of enzyme activity.  $A_0/A$  values thus obtained (where  $A_0$  was the initial activity before addition of clavulanate) were fitted to a single exponential.

## 3. RESULTS AND DISCUSSION

Qualitatively the three class C enzymes interacted with clavulanate in a similar way, with the following characteristics:

(i) Complete inactivation required  $C_0/E_0$  ratios ranging

from 50 to 150, which indicated the occurrence of branched pathways.

(ii) No irreversibly inactivated species appeared to accumulate, but the rates of reactivation were very low, as observed by Reading and Farmer [1].

(iii) In all cases, and in agreement with the partial inactivation results, turnover of clavulanate was detected.

In consequence, the results shown in Table I were interpreted on the basis of Scheme 1 by assuming that  $k_{-4}$  and  $k_6$  were negligible. The  $K_m$  values determined by monitoring the hydrolysis of clavulanate were similar to those determined in inactivation experiments with  $C_0/E_0 \gg k_3/k_{+4}$  and similarly, the  $k_3/k_{+4}$  ratios determined in the partial inactivation experiments were in good agreement with those deduced by comparing the  $k_{\text{cat}}/K_m$  and  $(k_i)_{\text{lim}}/K_m$  values. Fig. 1 shows the influence of clavulanate concentrations on the initial rates of clavulanate hydrolysis (1a) and on the pseudo-first order inactivation rate constants (1b) for the *Serratia marcescens*  $\beta$ -lactamase.

Our results with the *Enterobacter cloacae* 908R enzyme were also in good agreement with those of Reading and Farmer [1] ( $(k_i)_{\text{lim}}/K_m = 4\text{ M}^{-1} \cdot \text{s}^{-1}$ ,  $k_{\text{cat}} = 0.6 \times 10^{-4}\text{ s}^{-1}$ ) if one considers that their experiments were performed at  $37^\circ\text{C}$  and with a slightly different enzyme. The *Enterobacter cloacae* P99 and 908R enzymes differ by four residues [7].

Table I also compares the values of the kinetic parameters determined here with those for various class A enzymes. If one excepts the *Staphylococcus aureus* enzyme, the following striking differences can be highlighted:

(i) The  $k_{\text{cat}}/K_m$  values are at least three orders of magnitude lower for the class C enzymes.

(ii) This is, in part, due to the high  $K_m$  values observed with these latter proteins.

(iii) The  $(k_i)_{\text{lim}}/K_m$  are also distinctly lower with the class

Table I  
Kinetic parameters for the interactions with clavulanate

Source of enzyme	Class C $\beta$ -lactamases			Class A $\beta$ -lactamases	
	<i>Enterobacter cloacae</i> 908R	<i>Serratia marcescens</i> SC8247	<i>E. coli</i> K12	TEM I; <i>S. albus</i> G and S.R39 (extreme values)	<i>S. aureus</i>
$k_{\text{cat}} (\text{s}^{-1})$	$0.2 \pm 0.05$	$1 \pm 0.2$	$0.15 \pm 0.03$	3–50	$1.6 \times 10^{-4}$
$k_2/K' = k_{\text{cat}}/K_m (\text{M}^{-1} \cdot \text{s}^{-1})$	$40 \pm 10$	$200 \pm 40$	$17 \pm 3$	$4 \times 10^5$ – $10 \times 10^6$	4,000
$K_m (\text{mM})$	$5 \pm 2^a$	$5 \pm 1^a$	$9 \pm 2^a$	$0.7$ – $100 \times 10^{-3}$	N.D.
	$14 \pm 2^b$	$11 \pm 2^b$	$13 \pm 2^b$		
$(k_i)_{\text{lim}}/K_m (\text{M}^{-1} \cdot \text{s}^{-1})$	$1 \pm 0.4$	$2.5 \pm 0.5$	$0.7 \pm 0.2$	60–60,000	4,000
$(k_i)_{\text{lim}} (\text{s}^{-1})$	$0.014 \pm 0.003$	$0.026 \pm 0.004$	$0.009 \pm 0.002$	0.0024–0.09	N.D.
$(k_3/k_{+4} + k_6)$	$40 \pm 10^c$	$80 \pm 40^c$	$25 \pm 10^c$	150–20,000	N.D. (low)
	$50 \pm 10^d$	$150 \pm 40^d$	$70 \pm 20^d$		
$k_{\text{reac}} (\text{s}^{-1}) (= k_{-4} + k_5)$	$0.14 \pm 0.05 \times 10^{-4}$	$0.01 \pm 0.003 \times 10^{-4}$	$0.14 \pm 0.05 \times 10^{-4}$	$0.4 \times 10^{-4}$	N.D.

<sup>a</sup> From direct hydrolysis of clavulanate.

<sup>b</sup> From inactivation experiments.

<sup>c</sup> Calculated:  $(k_{\text{cat}}/K_m)/[(k_i)_{\text{lim}}/K_m]$ .

<sup>d</sup> From partial inactivation experiments.

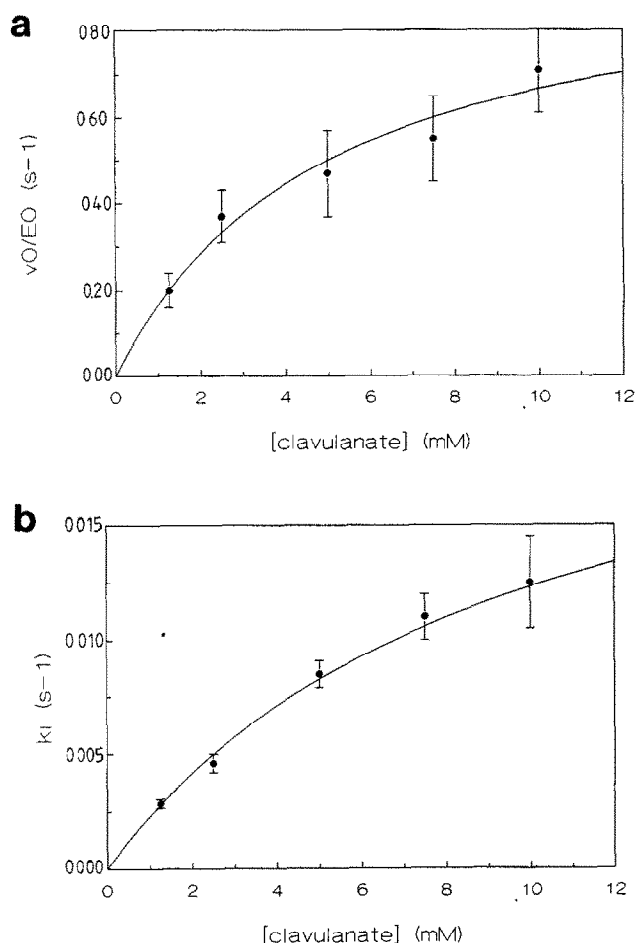


Fig. 1. Influence of the clavulanate concentrations on the initial rates of clavulanate hydrolysis (a) and on the rate of enzyme inactivation (b). ( $v_0/E_0$ ) were determined at medium  $C_0/E_0$  ratios (1000–10000) and extrapolated to  $[P] = 0$  as explained in the text. Pseudo-first order inactivation rate constants  $k_i$  were measured at high  $C_0/E_0$  ratios ( $> 100000$ ) with cefazolin as a reporter substrate [9].

C enzymes. Note that the lowest value in class A, obtained with the *Streptomyces albus* G  $\beta$ -lactamase, is due to a low  $(k_i)_{lim}$ , which is due to a very high  $k_3/k_{+4}$  ratio (20,000).

In consequence, it can be safely concluded that the poor efficiency of clavulanate as a class C inactivator mainly rests upon a relatively poor acylation rate of the enzyme, and is further decreased by a non-negligible turnover of this  $\beta$ -lactam ( $k_{cat} = 0.15\text{--}1\text{ s}^{-1}$ ). The  $K_m$  values are rather high and supply a minimum value for  $K'$  which characterises the  $[E][C]/[EC]$  ratio at the steady state.

The *Staphylococcus aureus* enzyme represents a

somewhat different situation. The  $k_2/K'$  value is relatively low ( $4,000\text{ M}^{-1} \cdot \text{s}^{-1}$ ) but the efficiency of the inactivation is increased by an exceedingly low  $k_3$  value. Indeed, with this enzyme  $k_{+4}$  does not appear to be very large and the inactivation is mainly due to accumulation of the first acyl-enzyme  $EC^*$  [8]. Here, the lower  $k_2/K'$  value is compensated by the nearly complete absence of turnover. The *Streptomyces albus* G enzyme supplies the other extreme example, with a high  $k_2/K'$  value ( $> 10^6\text{ M}^{-1} \cdot \text{s}^{-1}$ ) but as stated above, the turnover ( $k_3$ ) is much more efficient than the rearrangement step(s), resulting in the lowest  $(k_i)_{lim}/K_m$  obtained with a class A enzyme.

The properties of the *Staphylococcus aureus* enzyme also indicate that an efficient inactivation can rest on a rapid formation and high stability of  $EC^*$ , the first acyl-enzyme and that a high rearrangement rate is not necessary.

In consequence, if this rearrangement is slower with the class C enzymes, this fact is probably irrelevant to their resistance to clavulanate inactivation when compared to the influence of the low  $k_2/K'$  and high  $K'$  values. Moreover, the  $(k_i)_{lim}$  values only supply minimum values for  $(k_{+4} + k_6)$  and the data shown in Table I do not allow to suppose that the rates of acyl-enzyme rearrangement are specifically impaired with class C enzymes.

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