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# A rapid-kinetic study of the class C β-lactamase of *Enterobacter cloacae* 908R

Didier Monnaie[a], Richard Virden[b] and Jean-Marie Frère[a]

[a]*Laboratoire d'enzymologie, Université de Liège, Institut de Chimie, B6, B-4000 Sart-Tilman (Liège 1), Belgium* and [b]*Department of Biochemistry, University of Newcastle upon Tyne, Newcastle upon Tyne, UK*

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The individual rate constants for acylation and deacylation ($k_2$ and $k_3$, respectively) of the class C β-lactamase of *Enterobacter cloacae* 908R by ampicillin and carbenicillin have been determined. For several other β-lactams, the value of $k_2$ was too high to be determined and the $k_2/k_3$ ratio could be larger than 10,000. Branched pathways were also shown to occur with several penicillins and cephalosporins.

β-Lactamase: Cephalosporinase; *Enterobacter*; Penicillin; Cephalosporin; Rapid kinetics

## 1. INTRODUCTION

The production of β-lactamases is the major cause of bacterial resistance to β-lactam antibiotics and it can thus result in very serious clinical problems. A detailed understanding of the catalytic mechanism of these enzymes is thus a crucial step in the rational design of new penicillin analogues and related drugs which should ideally escape their hydrolytic action.

β-lactamases of classes A, C and D are active-site serine enzymes and in the simplest cases their catalytic pathway involves three steps:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES^* \xrightarrow{k_3} E + P \qquad \text{(Scheme I)}$$

where ES* represents the covalent acyl–enzyme intermediate [1], P the reaction product and where the steady-state parameters $k_{cat}$ and $K_m$ are related to the individual rate constants by the following equations:

$$k_{cat} = \frac{k_2 \cdot k_3}{k_2 + k_3},\ K_m = \frac{k_3 \cdot K'}{k_2 + k_3} \text{ and } K' = \frac{k_2 + k_{-1}}{k_1} \qquad (1, 2, 3)$$

A meaningful analysis of the modified enzymes obtained by site-directed mutagenesis requires a comparison of the individual rate constants. For several class A β-lactamases, these values have been determined with the wild-type and various modified enzymes [2]. By contrast, for class C β-lactamases chromosome-encoded enzymes which can be responsible for high level of resistance in Enterobacteria and related species, only the steady-state parameters are available [3,4] and in consequence, studies of modified enzymes remained rather superficial [5,6]. In the present communication, we describe the determination of the individual rate constants for a representative enzyme of class C, produced by *Enterobacter cloacae* 908R.

*Correspondence address:* J.-M. Frère, Laboratoire d'enzymologie, Université de Liège, Institut de Chimie, B6, B-4000 Sart-Tilman (Liège 1), Belgium. Fax: (32) (41) 563364.

## 2. MATERIALS, METHODS AND THEORETICAL BACKGROUND

The enzyme was produced and purified as before [7]. Nitrocefin was purchased from Oxoid (Basingstoke, Hants., UK), benzylpenicillin was from Rhône Poulenc (Paris, France), ampicillin was from Bristol Benelux S.A. (Brussels, Belgium), carbenicillin, cloxacillin, cefuroxime were from Glaxo Group Research (Greenford, Middx., UK), aztreonam was from the Squibb Institute for Medical Research (Princeton, NJ, USA), imipenem was from Merck, Sharp and Dohme Research Laboratories (Rahway, NJ, USA) cefotaxime was from Hoechst-Roussel (Romainville, France), temocillin and ticarcillin were from Beecham Research Laboratories (Brentford, Middx., UK) and carumonam was from Hoffman-LaRoche (Basel, Switzerland). All these compounds were gracious gifts from the various companies.

The formation of the acyl–enzyme was directly monitored at 260 nm for some cephalosporins (cefuroxime and ceftriaxone) but the reporter substrate method was utilised in most experiments. It was first verified that, within the dead time of the stopped-flow instrument (5 ms), a steady state was established with the reporter substrate, nitrocefin. Accumulation of the acyl–enzyme formed with the studied compound was then estimated by monitoring the decrease of the rate of nitrocefin hydrolysis, which was followed at 482 nm. Under these conditions and on the basis of Scheme I, the decay of the enzyme activity vs. nitrocefin was characterized by a pseudo first-order rate constant [8]:

$$k_a = k_3 + \frac{k_2 \cdot [C]}{K' \cdot \alpha + [C]} \qquad (4)$$

where C is the studied compound and α a correction factor allowing for the protection of the enzyme by nitrocefin ($\alpha = (K_m^n + [S])/K_m^n$, where $K_m^n$ is the $K_m$ of nitrocefin).

Individual $k_3$ values were obtained by monitoring the reactivation of a diluted sample after complete inactivation by aztreonam, imipenem and carumonam [3,4]. With ampicillin, carbenicillin,

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cloxacillin and cefotaxime, $k_3$ was determined by extrapolating to [C] = 0 the line obtained by plotting $k_a$ vs. [C] at low [C] values (see Eq. 4). Stopped-flow and quenched-flow experiments were performed at 30°C in 50 mM sodium phosphate buffer pH 7, containing 0.2 M NaCl with the help of a Bio-Logic SFM3 and a Bio-Logic QFM5 apparatus, respectively.

The acyl–enzymes formed with quinacillin and temocillin were assayed by monitoring the absorbance at 282 nm after addition of $HgCl_2$ [9]. This so called 'penamaldate reaction' results from a rearrangement of the penicilloyl moiety of the acyl–enzyme.

## 3. RESULTS

As shown in Fig. 1, formation of the acyl–enzyme intermediate followed saturation kinetics with carbenicillin and ampicillin and the individual K′ and $k_2$ values could be calculated (Table I). With several other compounds, no indication of saturation was observed for $k_a$ values lower than 150 $s^{-1}$ and only minimum values of $k_2$ and K′ could be derived (Table I). With a second set of compounds (ticarcillin, quinacillin, temocillin, cefuroxime, ceftriaxone, and ceftazidime) the rate of nitrocefin hydrolysis did not decrease according to a single exponential (Fig. 2) but could be fitted to the equation:

$$v_t - v^\infty = A \exp(-k_a t) + B \exp(-k_b t)$$

where $v_t$ and $v^\infty$ are the rates of nitrocefin hydrolysis at time $t$ and at the steady-state respectively (Table II).

This latter behaviour could be explained on the basis of Scheme I assuming a slow formation of the Henri–Michaelis complex ES. Alternatively, the presence of a potential leaving group on $C'_3$ of the three cephalosporins suggested the possibility of the branched pathway depicted by Scheme II [10] where ES** represents a second acyl–enzyme formed by the rearrangement of the cephalosporoyl moiety and P′ the product formed by hydrolysis of this latter acyl–enzyme.

$$E + S \underset{}{\overset{K'}{\rightleftharpoons}} ES \xrightarrow{k_2} ES^* \xrightarrow{k_3} E + P$$
$$\qquad\qquad\qquad\qquad\quad k_4 \uparrow\downarrow k_{-4} \quad k_5 \qquad \text{(Scheme II)}$$
$$\qquad\qquad\qquad\qquad\quad ES^{**} \dashrightarrow E + P'$$

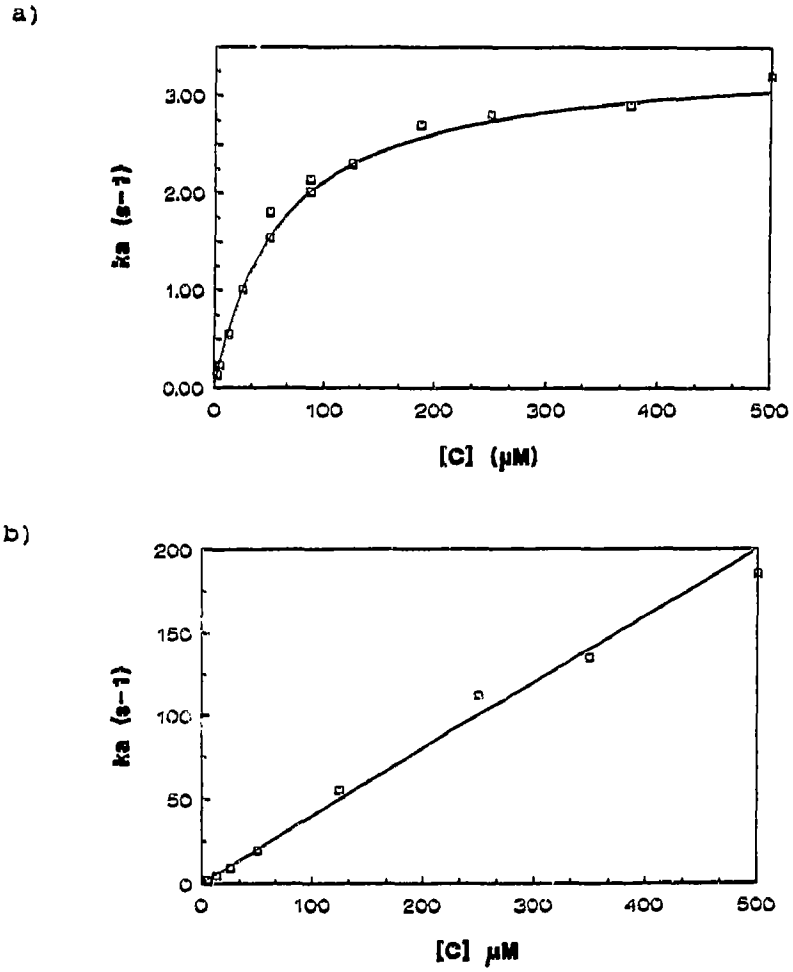


Fig. 1. Variation of the $k_a$ values with the inhibitory substrate concentration ([C]) for carbenicillin (a) and cefotaxime (b). The values are not corrected for the protection by the reporter substrate. The individual values of $k_2$ and $K'$ given in Table I were derived by fitting the results to the equations of a hyperbola (a) or a straight line (b).

Table I

Kinetic parameters for substrates exhibiting a linear pathway

| | $k_2$ ($s^{-1}$) | $k_3$ ($s^{-1}$) | $k_{cat}(\approx k_3)^f$ ($s^{-1}$) | $k_2/k_3$ | $K'$ ($\mu$M) | $k_2/K'$ ($\mu M^{-1}\cdot s^{-1}$) | $K_m^c$ ($\mu$M) | $k_{cat}/K_m$ ($\mu M^{-1}\cdot s^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| Carbenicillin | 3.3±0.2 | $3\pm1\times10^{-3}$ (a) | $4\pm1\times10^{-3}$ | 2,500 | 15±2 | 0.22±0.02 | (d) | (e) |
| Ampicillin | 120±30 | 1±0.2 (a) | 0.8±0.2 | 250 | 100±20 | 1.2±0.1 | 0.01±0.002 | 1.2±0.2 |
| Benzylpenicillin | >150 | N.D. | 15 | 10 | >5 | 30±5 | 0.6±0.1 | 35±10 |
| Cloxacillin | >150 | $6\pm2\times10^{-3}$ (a) | $4\pm1\times10^{-3}$ | >40,000 | >15 | 10±1 | (d) | (e) |
| Cefotaxime | >150 | $6\pm2\times10^{-3}$ (b) | $10\pm2\times10^{-3}$ | >25,000 | >100 | 0.5±0.02 | 0.010±0.004 | 1±0.2 |
| Imipenem | >150 | $3\pm1\times10^{-3}$ (b) | N.D. | >50,000 | >1,000 | 0.015±0.002 | (d) | (e) |
| Aztreonam | >150 | $2\pm1\times10^{-4}$ (b) | N.D. | $>7\times10^5$ | >800 | 0.220±0.03 | (d) | (e) |
| Carumonam | >100 | $7\pm2\times10^{-3}$ (b) | N.D. | >13,000 | >40,000 | 0.080±0.005 | (d) | (e) |

(a) measured by reactivation (by stopped-flow for ampicillin).
(b) measured by extrapolation.
(c) measured as $K_i$ values.
(d) $K_m$ cannot be independently determined.
(e) $k_2/K'$ was the only measured value.
(f) It can be seen that $k_{cat}$ is ($\ll$ than $k_2$ in all cases so that one can safely assume that $k_{cat} \approx k_3$ (see Eq. 1). Moreover for ampicillin, cloxacillin, carbenicillin and cefotaxime the values are in good agreement with the directly determined $k_3$ values.

In Scheme II, the E + S $\rightleftharpoons$ ES equilibrium is immediately established and the two exponentials arise from the successive accumulations of ES* and ES**. In a first approximation one can consider that the first and second exponentials respectively characterize the accumulation of ES and ES* in Scheme I and ES* and ES** in Scheme II.

To distinguish between the two models, we tried to determine if the cephalosporin was covalently bound to the enzyme after a time long enough to accumulate the first complex (ES in Scheme I and ES* in Scheme II) but too short to allow a significant accumulation of the second. Stoichiometric amounts of enzyme and ceftriaxone were mixed in the quenched-flow apparatus (final concentrations: 50 $\mu$M) and the reaction was quenched after 0.5 s by addition of 1 N HCl. The acidification resulted in the precipitation of the protein, and no free ceftriaxone was found in the supernatant as shown by the absence of material absorbing at 330nm a wavelength at which ceftriaxone exhibits an $\varepsilon$ value of 1,500 $M^{-1}\cdot cm^{-1}$, thus indicating the validity of Scheme II in this case. Indeed one would expect free ceftriaxone to be regenerated upon acid denaturation of the non-covalent ES complex in Scheme I. Technical difficulties did not allow us to perform similar experiments with the two other compounds, but it seems unlikely that they would behave differently.

The biphasic inhibition kinetics observed with quinacillin, ticarcillin, and temocillin also seemed to be due to a branched pathway. Indeed, when the reaction between 15 $\mu$M enzyme and 200 $\mu$M quinacillin or temocillin was stopped after 15 s the penamaldate assay indicated that the acyl–enzyme was already formed before the second phase of the reaction. In these cases, no

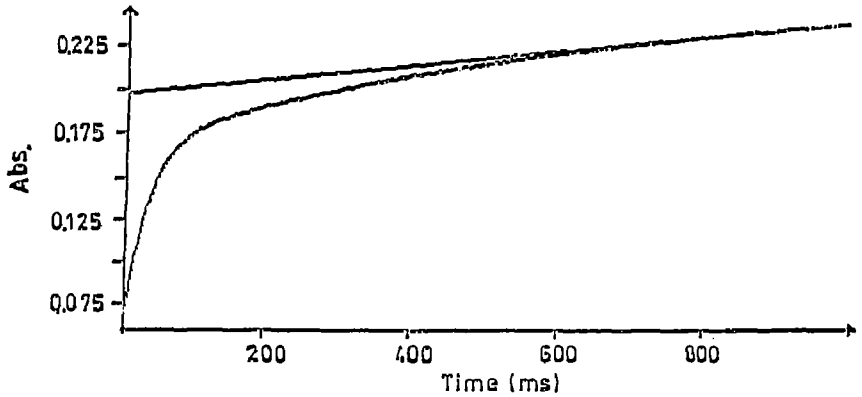


Fig. 2. Biphasic inhibition by 25 $\mu$M cefuroxime monitored by the decrease of the rate of nitrocefin followed at 482 nm. The line represents the steady-state rate of nitrocefin hydrolysis. Fitting was significantly better when using a sum of two exponential than a single one (the sum of the squared deviations being 10-fold lower in the former case than in the latter).

Table II

Kinetic data for the interactions with compounds yielding biexponential inactivation time-courses

| | [C] ($\mu$M) | $k_a$ ($s^{-1}$) | $k_b$ ($s^{-1}$) | A/B |
|---|---|---|---|---|
| Cefuroxime | 12.5 | 14 | 1.7 | 0.76 |
| Ceftriaxone | 25 | 12 | 0.54 | 1.5 |
| Ceftazidime | 1250 | 1.1 | 0.25 | 1.6 |
| Temocillin | 50 | $23\times10^{-3}$ | $8\times10^{-3}$ | N.D. |
| Ticarcillin | 375 | 27 | 4 | 0.54 |
| Quinacillin | 250 | 1.4 | $4\times10^{-3}$ | N.D. |

modification of the substrate moiety could be implicated, so that a conformational change of the enzyme itself appears to be likely.

## 4. DISCUSSION

With several class A $\beta$-lactamases, the $k_2/k_3$ ratios have been obtained by estimating the proportions of acyl-enzyme at the steady-state with the help of the penamaldate method, and the values of the two constants were of the same order of magnitude [2]. Methanolysis experiments performed with another class C enzyme [1] indicated acylation to be faster than deacylation. The results obtained in the present study show that with the *Enterobacter cloacae* 908R $\beta$-lactamase, $k_2$ is always much larger than $k_3$ and that the $k_2/k_3$ ratio can be extremely high. With several substrates, the behaviour of this enzyme is reminiscent of that of the penicillin-sensitive DD peptidases, a rapid acylation followed by a slow or very slow deacylation leading to the transient immobilization of the protein as an acyl–enzyme. In this respect, the behaviour of this class C enzyme thus sharply contrasts with those of the class A $\beta$-lactamases.

It is important to note that the $k_2/K'$ values determined by monitoring the accumulation of the acyl–enzyme were in good agreement with the $k_{cat}/K_m$ ratios computed from the $k_{cat}$ values (obtained by directly measuring the hydrolysis of the $\beta$-lactams) and from the $K_m$ values, measured as $K_i$ values in competition experiments at the steady state. Such an agreement between a value ($k_2/K'$) determined according to a procedure where the exact knowledge of the enzyme concentration is not necessary, and another one ($k_{cat}/K_m$) for which that concentration is directly involved in the computation of $k_{cat}$, demonstrates that most of the enzyme molecules present in the preparation used in the latter experiments were actually active and that the preparation was pure or nearly so.

Our study thus supplies the first individual values of $K'$, $k_2$ and $k_3$ for a class C $\beta$-lactamase and can serve as a basis for the analysis of the properties of modified proteins obtained by site-directed mutagenesis. It should however be noted that the best cephalosporin substrates of the *Enterobacter cloacae* $\beta$-lactamase exhibit $k_{cat}$ values larger than 200 $s^{-1}$ [4]. Thus, with these compounds, it was not possible to measure the accumulation of the acyl–enzyme, since the steady-state was reached within the mixing dead-time of the apparatus.

The inactivation of the enzyme monitored with nitrocefin as a reporter substrate was biphasic with several cephalosporins (cefuroxime, ceftriaxone and ceftazidime) with which a rearrangement of the cephalosporoyl moiety of the acyl-enzyme could be suspected involving the elimination of the C3′ leaving group as proposed by Mazzella and Pratt [10]. The similar behaviour observed with some penicillins might be related with a conformational change such as that described by Citri et al. [11]. Such a behaviour is particularly misleading since the second step shows saturation kinetics at high inhibitor concentration and thus could be interpreted as a saturation at the level of the Henri–Michaelis complex if no attention is paid to the early phase.

These observations underline a potential major difficulty in the analysis of the influence of mutations on the activity of class C enzymes. If the occurrence of branched pathways can be generalized to all cephalosporins containing a potential leaving group on $C'_3$, the interpretation of variations of the steady-state parameters $k_{cat}$ and $K_m$ will become quite complicated. Moreover, it seems that branched pathways are also prevalent with some penicillins. Class C enzymes generally behave as a very homogenous family [3,4] and qualitatively, our conclusions are probably valid for most if not all members of this group.

Finally the present study also demonstrates the strength of the reporter substrate method when applied to the study of mediocre and poor substrates. Indeed, it allows the determination of the kinetic parameters of these compounds with relatively small quantities of enzyme, even when rapid kinetic methods must be utilised.

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