Biochemical Pharmacology, Vol. 30, pp. 549–552.
Pergamon Press Ltd. 1981. Printed in Great Britain.

0006-2952/81/060549-04 $02.00/0

COMMENTARY

INTERACTION BETWEEN SERINE β-LACTAMASES AND CLASS A SUBSTRATES: A KINETIC ANALYSIS AND A REACTION PATHWAY HYPOTHESIS

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Fifty years after Fleming, penicillins and Δ^3-cephalosporins are probably the most widely used antibacterial agents. Unfortunately, many pathogenic strains have emerged, which possess a very efficient defence against these chemotherapeutic compounds: these bacteria synthesize one, sometimes several β-lactamases, which are extremely efficient penicillin- and (or) cephalosporin-destroying enzymes. The products of the action of β-lactamases on penicillins are penicilloic acids, substances devoid of antibacterial properties. It is thus not surprising that β-lactamases have received the attention of an increasing number of biochemists.

The study of the structure of the β-lactamases, and of their interactions with their substrates has yielded a vast number of results. Some of these are of particular interest to the present commentary: certain β-lactams, referred to as "Class A substrates" by Citri *et al.* [1], were not only rather poor substrates of β-lactamase I from *Bacillus cereus* and of the β-lactamase from *Staphylococcus aureus*, but they were also capable of conferring on these enzymes a conformation which was less active towards the usual good substrates [1, 2] (Class S substrates). Nonetheless, besides these rather unusual observations, the mode of action of these enzymes remained, until recently, rather mysterious. Within the last few months, however, a surprisingly large number of relevant results has been collected. First, highly specific inactivators were found, some of them behaving as poor substrates before the inactivation was completed: β-bromopenicillanic acid, clavulanate, derivatives of olivanic acid and sulfones of quinacillin and penicillanic acid (for recent reviews, see [3–5]).

Using one of these inactivators, which could be more accurately described as suicide substrates, Knott-Hunziker *et al.* [6] performed the first affinity labeling of the active site of β-lactamase I from *B. cereus*. Proteolysis of the enzyme, after reaction with tritiated β-bromopenicillanic acid, demonstrated that the inactivator was ester-bound to the side-chain of Serine 44. As shown by Ambler [7], this residue is conserved in the four β-lactamases whose primary structures are known (*B. cereus I, B. licheniformis, S. aureus* and *Escherichia coli* RTem). Similarly, Knowles and his co-workers (personal communication) isolated, from the RTem enzyme, a peptide labeled with quinacillin sulfone. This peptide also contained residue Serine 44, and it seems very likely that the reagent is also ester-bound to the side chain of this residue. β-lactamase II from *B. cereus* is completely different: it contains one essential Zn^{2+} ion [8] and is not inactivated by clavulanate or the other suicide substrates of β-lactamase I.

The discovery of an essential serine residue in an important class of β-lactamases had been preceded by the demonstration that, with the penicillin sensitive DD-carboxypeptidases–transpeptidases from *Streptomyces* R 61 and *Actinomadura* R 39 and DD-carboxypeptidases from *Bacillus stearothermophilus* and *Bacillus subtilis*, the interaction between enzyme and penicillins involved the hydroxyl group of a serine residue [9–12]. The inactive complex thus formed slowly decayed, regenerating active enzyme and yielding a fragmented penicillin molecule: *N*-acylglycine and *N*-formylpenicillamine were identified when either benzylpenicillin or phenoxymethylpenicillin were used [13–14]. Thus, these enzymes behaved as penicillin-destroying enzymes of exceedingly low efficiency: k_{cat} ranged from less than 10^{-6} to somewhat more than $10^{-3}\ s^{-1}$. The model which was proposed for the interaction between penicillins and these enzymes involved the formation of a relatively stable intermediate complex [15, 16] (scheme 1).

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} EI^* \xrightarrow{k_3} E + P(s)$$

Scheme 1. Interaction between penicillins (I) and penicillin-sensitive DD-Carboxypeptidases–transpeptidases (E).

When it could be separated from steps 2 and 3, the first step was always found to equilibrate rapidly (with $k_{-1}/k_1 = K$ = dissociation constant of EI). The second and third steps were irreversible, with $k_2 \gg k_3$, which explained the immobilization of a very large proportion of the enzyme as the inactivated form EI*. Although the direct demonstration of the presence of the ester bond in a native complex formed between enzyme and inhibitor still remains to be performed, n.m.r. results obtained after denaturation of the complex formed between benzylpenicillin and the DD-carboxypeptidase–transpeptidase from *Streptomyces* R 61 indicated that EI* was very probably a penicilloyl ester of the serine side-chain [17].

The results obtained with the serine β-lactamases and their suicide substrates suggest a similar mechanism for these latter enzymes. Inactivation occurs when the acylenzyme can irreversibly rearrange into a stable complex [5]. Although it might be dangerous

to extrapolate to good substrates a mechanism which has been found valid for poor or suicide substrates, it seems likely that scheme I also depicts the interaction between the serine β-lactamases and their substrates. Contrary to what was observed with penicillin-sensitive enzymes, the value of k_3 would naturally be large, but the distinction between the two types of enzymes might become arbitrary: the value of k_{cat} can be as low as 0.2–2 s^{-1} for some poor substrates of β-lactamases and a value of 0.3 s^{-1} has been found for the spontaneous degradation of the EI* complex formed between benzylpenicillin and the membrane DD-Carboxypeptidase from *S. aureus* [18].

When the interaction between β-lactamases and good substrates has been studied, the usual kinetic parameters, k_{cat} and K_m have been determined. For a reaction obeying scheme 1, these parameters will be respectively equal to $k_2k_3/(k_2+k_3)$ and $k_3K/(k_2+k_3)$ (general equations). Obviously, $k_{cat}/K_m = k_2/K$. For penicillin-sensitive enzymes, k_3 and k_2/K are usually measured. The individual values of k_2 and K have only been obtained in some favourable cases.

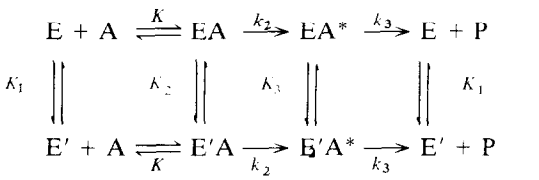
Actinomadura strain R39 produces both a β-lactamase and a penicillin-sensitive DD-Carboxypeptidase–transpeptidase [19, 20]. Inactivation of the latter enzyme by benzylpenicillin involves the acylation of a Serine residue [10].

It was not demonstrated that the β-lactamase was a Serine enzyme but it exhibited no requirement for a divalent cation and it was inactivated by clavulanate, cefoxitin and quinacillin sulfone [21, 22]. The values of k_{cat}/K_m for the β-lactamase were compared to those of k_2/K for the DD-Carboxypeptidase. For 7 penicillins and 4 cephalosporins, (values ranging from 4×10^3 to 10×10^6 $M^{-1}s^{-1}$), a surprisingly good agreement was found [21]. Although the agreement was poor for cephalosporin C and very poor for 6-aminopenicillanic acid, these data indicated a possible similarity in the acylation step catalyzed by both enzymes. Deacylation was rapid ($k_3 \gg 4\ s^{-1}$) with the β-lactamase and exceedingly slow ($k_3 \simeq 10^{-6}\ s^{-1}$) with the DD-Carboxypeptidase. All these data strongly suggest that the hydrolysis of penicillins and cephalosporins, when catalyzed by the Serine-β-lactamases also implies an acylation–deacylation mechanism.

Citri *et al.* [1] observed that the hydrolysis of Class A substrates (substrates with a large, sterically hindered side-chain) in the presence of the *B. cereus* β-lactamase I was a biphasic phenomenon. For all Class A substrates, the values of k_{cat} and K_m were lower in the second than in the first phase, but the ratio k_{cat}/K_m was preserved. The "uncompetitive inhibition" of the same enzyme which was recorded by Strom *et al.* [23] after preincubation with isoxazolylpenicillins (which are Class A substrates) had essentially the same meaning: decreased values of k_{cat} and K_m accompanied by an unaltered ratio k_{cat}/K_m.

Virden *et al.* [2] recorded a similar behavior of the β-lactamase from *S. aureus*, using quinacillin as substrate: the values of K_m and V were both decreased in the second phase, but the ratio k_{cat}/K_m remained unchanged.

A widely accepted explanation of these phenomena was that these substrates induced the appearance of a new, less active conformer of the enzymes. Since in a mechanism such as that described by scheme 1, k_{cat}/K_m equals k_2/K, these results mean that the conformational transition does not affect k_2/K. If one assumes that acylation is rate-limiting, ($k_{cat} = k_2$; $K_m = K$), the experimental observations can only be explained if k_2 and K are modified by the same factor, a very unlikely situation. On the contrary, if deacylation is rate-limiting ($k_{cat} = k_3$; $K_m = k_3K/k_2$) or if both steps occur at similar rates (general equations), a decrease of the single constant k_3 explains both the decrease of k_{cat} and K_m by a similar factor and, thus, the stability of k_{cat}/K_m. This does not mean that K and k_2 would remain completely unchanged, but the variation of the kinetic constants would reflect mainly the variation of k_3. This hypothesis can be summarized by scheme 2.



Scheme 2. Possible mechanism of interaction between a Class A substrate (A) and the serine β-lactamases from *B. Cereus* and *S. aureus*. All the steps represented by vertical arrows are *slow*.

$$K_1 = \frac{(E)}{(E')},\ K_2 = \frac{(EA)}{(E'A)},\ K_3 = \frac{(EA^*)}{(E'A^*)}$$

According to our hypothesis, $k_2 \simeq k_2'$, $K \simeq K'$ and $k_3 > k_3'$. K and K' are the dissociation constants of EA and E'A, respectively.

Before the substrate is added, E is in equilibrium with E' (K_1). Upon addition of a class A substrate, the initial velocity (v_0, the initial velocity observed by Citri *et al.*) is the sum of two essentially independent reactions: one catalyzed by E and one by E'. The reactions EA ⇌ E'A and EA* ⇌ E'A* then slowly attain equilibrium and the system reaches the steady-state (v_f, the final phase observed by Citri *et al.*).

A mathematical analysis of the model shows that the equation $v_0 = f(A)$ is not of the Henri–Michaelis type. However, if one introduces the following additional restrictions

(1) E is the predominant form in the absence of substrate (i.e. $K_1 > 1$),

(2) v_0 is measured with concentrations of S not very different from the apparent K_m,

(3) $k_3' < k_3$,

one can show by simulation that within the limits of experimental errors, plots of $1/v$ versus $1/(A)$ yield straight lines from which apparent K_m's and V's can easily be computed. Not surprisingly, these values are somewhat lower than the values which would be obtained if only the upper branch of the scheme were operative, and the ratio k_{cat}/K_m is close to k_2/K. It is thus nearly impossible to distinguish between such a system and a simple, one enzyme mechanism from a Lineweaver–Burk plot.

At the steady-state, the value of k_{cat}/K_m depends upon K, K', k_2, k_2', k_3, k_3', and K_3. But if $k_2 \simeq k_2'$ and

$K \simeq K'$, this ratio again becomes equal to k_2/K. The individual values of k_{cat} and K_m are then smaller than the values of the corresponding apparent constants at $t = 0$. In this model, the decrease in reaction velocity is due to the progressive immobilization of the enzyme in the form E'A*. This form would be more sensitive to inactivating agents [24] than form EA*. Conversely, form E, and the intermediates EA and EA* would be stabilized in the presence of antibodies [25]. An interesting simplification of the general scheme is given by scheme 3.

$$E + A \underset{K}{\overset{\text{fast}}{\rightleftharpoons}} EA \xrightarrow{k_2} EA^* \xrightarrow{k_3} E + P$$

$$EA^* \underset{K_3, \text{slow}}{\rightleftharpoons} E'A^*$$

Scheme 3. E'A* is the only representative of enzyme form E' and is non-productive ($k_3' = 0$).

This scheme is based on a suggestion made by Hill, Sammes and Waley [4]: "For some substrates, the acyl-enzyme is prone to unfolding". At the steady-state K_m and k_{cat} are divided by the same factor:

$$\frac{k_3 + k_2[1 + (1/K_3)]}{k_2 + k_3}$$

which is always >1. Obviously, k_{cat}/K_m remains unchanged. The common corollary to both schemes is that the progressive inactivation of the enzyme, corresponding to the shift from the active (E) to the less active or inactive (E') conformation, is due to the accumulation of E'A*.

In the interaction between clavulanate and the RTem β-lactamase, Fisher *et al.* [26] have postulated the transient appearance of a relatively stable complex (designated as E-t by these authors) which would correspond to complex E'A*. Nearly complete, slowly reversible inactivation, such as that recorded by Sagai and Saito [27] can be explained, either by a very stable complex EA* (small k_3) or by a very stable complex E'A* (small k_3' and small K_3).

The interpretation of the substrate induced inactivations according to scheme 2 or 3 rests upon the assumption that acylation of the enzyme is not the rate-limiting step.

The transient formation of an acylenzyme, followed by a rate-limiting deacylation is well documented in the chymotrypsin-catalysed hydrolysis of esters [28]. However, the catalysis of the hydrolysis of amides by the same enzyme is less clearly understood: if any acyl enzyme is formed, it remains undetectable because deacylation is much faster than acylation. This latter step occurs at a much lower rate than with esters: the ratios k_3/k_2 are 900 and 2000 for the amides of *N*-acetyltryptophane and *N*-acetyl-phenylalanine, respectively [29]. For the methyl esters and the amides of the same *N*-acetyl-amino acids, the ratios (k_2) methyl ester/(k_2) amide are 21.000 and 12.500 and, in consequence, the ratios (k_{cat}') methyl ester/(k_{cat}) amide are 1000 and 1600, representing, in fact, the ratios (k_3) methyl ester/(k_2) amide. For the amides, deacylation is as stated above, 1000–2000 times faster than acylation, whereas for the esters, acylation is 4–20 times faster than deacylation.

However, penicillins and Δ^3-cephalosporins are much more susceptible to nucleophilic attack than linear amides. Table 1 summarizes the susceptibility of various amide and ester bonds to nucleophilic attack by OH^- ions (k_{OH^-}): the hydrolysis of penicillins and Δ^3-cephalosporins proceeds 10^3 to 10^5 times faster than that of linear amides. In fact, there is nearly no difference between the rate of hydrolysis of benzyl-penicillin (I) and that of α-Methylpenicilloate (II).

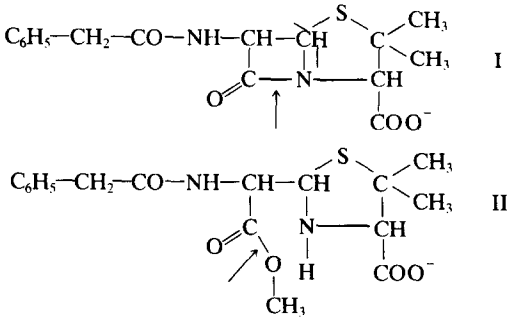


Table 1. Susceptibility of various amide and ester bonds to nucleophilic attack by OH^- ions

	Compound	k_{OH^-} ($M^{-1}s^{-1}$)	Temp.	Ref.
A	Penicillins	0.1–1	25°	30
	Δ^3-cephalosporins			
	Fused, planar β-lactams	10^{-3}–10^{-2}	25°	30
	Non-fused β-lactams	10^{-6}–10^{-5}	25°	30
	Linear amides			
B	Ethyl acetate	0.1	25°	31
	Methyl acetate	0.2	25°	29
	Acetamide	4×10^{-5}	25°	29
C	Benzylpenicillin	0.47	37°	†
	α-Methylbenzylpenicilloate	0.73	37°	†

† [^{14}C]-α-Methylbenzylpenicilloate or [^{14}C]benzyl-penicillin were incubated at 37° in 0.1 M potassium phosphate buffer, pH 11.8. Samples (5–10 μl) were withdrawn after increasing periods of time (1 to 15 min) and submitted to t.l.c. in solvent I. Benzyl penicillin ($R_f = 0.65$) and α-methylpenicilloate ($R_f = 0.57$) were well separated from penicilloate ($R_f = 0.05$). The radioactivity on the plates was quantified with the help of a Packard 2000 Radiochromatogram scanner. The area under each peak was estimated by triangulation.

[^{14}C]-α-Methylbenzylpenicilloate was prepared by incubating 5 nmoles of [^{14}C]benzylpenicillin (50 mCi/nmole, Amersham) in absolute methanol at 37° for 240 min. Progress of the reaction was followed by adding β-lactamase to samples and determining the amount of penicilloate formed by t.l.c. in solvent I. After completion of the reaction, the identity of the radioactive product was demonstrated by thin-layer co-chromatography with authentic cold α-methylbenzylpenicilloate (a gift from Dr. B. Meesschaert, Rega Instituut, K. U. L., Leuven, Belgium) in solvents I and II. After drying of the plates, α-methylpenicilloate was revealed by spraying the plate with an alcoholic, neutralized solution of bromocresol green (solvent I: chloroform, methanol, acetic acid: 88:10:2, v/v; solvent II: 1 butanol, H_2O, acetic acid, ethanol: 10:4:3:3, v/v. Thin layer chromatograms were developed on Polygram Sil G Plates—Macherey—Nagel).

Conversely, the rate of hydrolysis of α-methylpenicilloate is quite similar to that of methyl acetate. During an enzyme-catalysed hydrolysis involving a nucleophilic attack, one can therefore expect the behaviour of benzylpenicillin to be closer to that of an ester than to that of a linear amide. If the acylation–deacylation pathway is utilized by the Serine β-lactamases, it is likely that acylation of the enzyme by the β-lactam is not the rate-limiting step. From this analysis one can conclude that the biphasic time–course observed during the hydrolysis of type A substrates by the Serine β-lactamases from *S. aureus* and *B. cereus* is probably due to progressive immobilization of the enzyme as complex E′A*. It appears that these two enzymes are rather "floppy" molecules [32] which easily undergo conformational changes. On the contrary, the RTem β-lactamase, which is also a serine enzyme, has a more rigid structure and has not been shown, as yet, to undergo a conformation change in the presence of class A substrates. It is also important to note, at this point of the discussion, that biphasic kinetic responses have never been observed with the Zn^{2+} β-lactamase II from *B. cereus*, an enzyme for which it is difficult to imagine a reaction pathway involving an acylation-deacylation sequence.

In conclusion, the present hypothesis on the mechanism of hydrolysis of penicillins by Serine β-lactamases can be summarized as follows:

(1) The reaction pathway involves acylation of the active site Serine side-chain by the antibiotic, followed by the hydrolysis of the newly-formed ester bond.

(2) The acylation is not the rate-limiting step of the reaction: either acylation and deacylation proceed at similar rates, or deacylation is slower.

(3) In some "floppy" enzymes, Class A substrates induce or stabilize a conformation which is different from that which predominates in the absence of substrates or in the presence of Class S substrates; with Class A substrates, the decrease of both k_{cat} and K_m can be attributed to the progressive accumulation of E′A*, an acylenzyme which is hydrolyzed more slowly than EA* ($k_3' < k_3$).

Acknowledgements—The author wishes to thank Drs P. E. Reynolds and J. M. Ghuysen for interesting discussions. This work was supported by the *National Institutes of Health*, U.S.A. (contract n°2 R01 AI13364-04), the *Fonds de la Recherche Scientifique Médicale*, Brussels (contract n°3.4501.79) and the *Actions Concertées*, Brussels (convention n°79/84-11).

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Note added in proof.—Serine residues were recently found in the active sites of β-lactamases of *S. aureus* (S. J. Cartwright and A. F. W. Coulson, *Phil. Trans. R. Soc. Lond. B* **289**, 370, 1980) and of *Pseudomonas aeruginosa* (V. Knott-Hunziker, K. Redhead, S. Petursson and S. G. Waley, *FEBS Lett.*, in press).

Experiments with the β-lactamase I of *B. cereus* and two Class A substrates (cloxacillin and methicillin) clearly indicated that the substrate-induced inactivation was due to the reversible accumulation of an acyl-enzyme intermediate exhibiting very little or no catalytic competence (P. A. Kiener, V. Knott-Hunziker, S. Petursson and S. G. Waley, *Eur. J. Biochem.* **109**, 575, 1980). Whether the absence of inactivation with Class S substrates was due to an increased stability of intermediate ES* (high K_3) or only to kinetic factors ($k_2 \gg k_3$) remains to be determined. This problem could be solved by a direct estimation of constants k_2 and k_3 for a good Class S substrate.