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Mode of Interaction between β-Lactam Antibiotics and the Exocellular DD-Carboxypeptidase–Transpeptidase from *Streptomyces* R39

By NOHA FUAD, JEAN-MARIE FRÈRE, JEAN-MARIE GHUYSEN and COLLETTE DUEZ
Service de Microbiologie, Faculté de Médecine, Institut de Botanique, Université de Liège, Sart Tilman, 4000 Liège, Belgium

and MOTOHIRO IWATSUBO
Centre de Génétique Moléculaire, C.N.R.S., 91190 Gif-sur-Yvette, France

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The exocellular DD-carboxypeptidase–transpeptidase of *Streptomyces* R39 is inhibited by β-lactam antibiotics according to the same general scheme of reaction as the exocellular DD-carboxypeptidase–transpeptidase of *Streptomyces* R61. However, the values for the kinetic constants involved in the reaction are very different for the two enzymes, and provide an explanation for the observation that the R39 enzyme is more sensitive to β-lactam antibiotics than the R61 enzyme. Further, particular β-lactams influence the kinetic constants to different extents depending on the source of the enzyme, so that a physical basis for the spectrum of antibiotic activity against particular enzyme systems is provided.

Both exocellular DD-carboxypeptidases–transpeptidases from *Streptomyces* R61 and R39 react with penicillins and cephalosporins to form equimolar and inactive enzyme–antibiotic complexes (Frère *et al.*, 1974*b*,*c*). When incubated in the absence of free antibiotic under conditions in which the enzyme is stable, the complexes are broken down with concomitant recovery of the enzymic activity and the penicillin sensitivity of the enzyme. However, the released antibiotic is fragmented. With benzylpenicillin, one of the fragments is phenylacetylglycine (Frère *et al.*, 1975*a*). The simplest model that best explains the interaction between the R61 enzyme and β-lactam antibiotics involves three steps:

$$E+I \underset{}{\overset{K}{\rightleftharpoons}} EI \xrightarrow{k_{+3}} EI^* \xrightarrow{k_{+4}} E+X+Y$$

(1) formation through a rapid equilibrium process of the equimolar complex EI;† (2) isomerization into a modified complex EI*; (3) breakdown of this latter complex with the release of an active enzyme and antibiotic fragments X and Y (Frère *et al.*, 1975*b*). The k_{+4} values were determined on the basis of the half-lives of the complexes EI* (Frère *et al.*, 1974*b*,*c*), and the K and k_{+3} values were estimated by studying the effects of inhibitor concentration on the values of the apparent rate constant for the formation of complex EI*, i.e.

$$k_a = k_{+3} \Big/ \left(1+\frac{K}{[I]}\right)$$

(Frère *et al.*, 1975*b*). At low antibiotic concentrations, the plots k_a versus [I] are linear; they extrapolate to the origin and the slopes of the lines give the ratio k_{+3}/K. At high antibiotic concentrations, non-linearity is observed and, under these conditions, the double-reciprocal plots $1/k_a$ versus 1/[I] give rise to lines that intercept the ordinate axis at the $1/k_{+3}$ values and the slopes of which are equal to the ratio K/k_{+3}. In all those experiments, the condition $[E] \ll [I]$ was fulfilled. This condition is necessary to allow linearization of the differential equations and integration of the rate equation used for the mathematical treatment of the model (Frère *et al.*, 1975*b*).

The interaction between the exocellular DD-carboxypeptidase–transpeptidase of *Streptomyces* R39 and penicillins and cephalosporins has been studied. The present paper reports results of experiments that show that the interaction of β-lactam antibiotics and the R39 enzyme are compatible with the mechanism that had been described for the R61 enzyme. However, there is a large variation in the values of the constants K, k_{+3} and k_{+4} between the two enzymes.

† Abbreviations: E, active enzyme; I, inhibitor (i.e. a β-lactam antibiotic); EI, an intermediate and inactive enzyme–inhibitor complex; EI*, inactive enzyme–inhibitor complex in which both constituents are modified; X, released and chemically modified antibiotic; k_a, apparent rate constant for the formation of complex EI* in the absence of substrate; k'_a, apparent rate constant for the formation of complex EI* in the presence of substrate.

Materials and Methods

Enzyme R39 DD-carboxypeptidase–transpeptidase and measurement of enzyme activity

Purified R39 enzyme was used [specific activity, 18 units/mg of protein (Frère *et al.*, 1974*a*)]. One unit hydrolyses 1 μmol of Ac_2-L-Lys-D-Ala-D-Ala to D-alanine and Ac_2-L-Lys-D-Ala (DD-carboxypeptidase activity) per min at 37°C and under conditions of saturation of the enzyme by the substrate (the K_m value is 1 mM in 0.1 M-Tris/HCl buffer, pH 7.7, containing 0.1 M-NaCl and 0.05 M-$MgCl_2$). The enzyme activity was measured by incubating Ac_2-L-Lys-D-Ala-D-Ala (3 mM, final concn.) with the enzyme at 37°C and in 35 μl (final vol.) of the Tris/NaCl buffer supplemented with 0.02 M-$MgCl_2$. Free D-alanine was measured enzymically as described by Johnson *et al.* (1975). The enzyme preparation used was contaminated by a trace of penicillinase (0.7 munit/mg of protein).

β-Lactam antibiotics

Benzylpenicillin (UCB, Brussels, Belgium), ampicillin (Bristol Benelux, S.A., Brussels, Belgium), methicillin and carbenicillin (Beecham, S.A., Brussels, Belgium), cephaloglycine, cephalexin and cephalosporin C (Lilly Laboratories, Indianapolis, IN, U.S.A.) and cephalosporin 87-312 [3-(2,4-dinitrostyryl)-(6*R*-7*R*)-7-(2-thienylacetamido)-ceph-3-em-4-carboxylic acid, E isomer; Glaxo Research, Greenford, Middx., U.K.] were used. Concentrated antibiotic solutions (10 mM) were made up in water, except that of cephalosporin 87-312, which was made in dimethylformamide (from which appropriate dilutions were made in 10 mM-sodium phosphate, pH 7.0; the final concentrations being estimated on the basis of a molar extinction coefficient at 386 nm $\varepsilon = 17500$ litre·mol^{-1}·cm^{-1}).

Penicillinases

Riker penicillinase was used for the hydrolysis of the various penicillins and of cephalosporin 87-312. The exocellular penicillinase of *Streptomyces* R39 (specific activity, 200 units/mg of protein) (Johnson *et al.*, 1975) was used for the hydrolysis of the other cephalosporins. One unit of penicillinase hydrolyses 1 μmol of benzylpenicillin per min either at 25°C (Riker penicillinase) or at 30°C (*Streptomyces* R39 penicillinase).

Buffers

In all cases, the interaction between the R39 enzyme and the various β-lactam antibiotics was studied in 0.1 M-Tris/HCl buffer, pH 7.7, containing 0.1 M-NaCl and 0.05 M-$MgCl_2$, under which conditions the R39 enzyme exhibits high stability. For the estimation of DD-carboxypeptidase activity, the reaction mixtures were diluted so that the concentration of $MgCl_2$ was decreased to 0.02 M. Concentrations of $MgCl_2$ greater than this cause turbidity in the reaction mixtures used for the enzymic determination of D-alanine.

*Determination of the rate constants (k_{+4}) for the breakdown of complexes EI**

The values obtained previously with some β-lactam antibiotics and at 37°C were used (Frère *et al.*, 1974*c*), and the values for the others were determined by the same procedure. Enzyme (66 μM) and antibiotic (400 μM) were incubated together at 37°C for 12 min in 60 μl (final vol.) of buffer. The excess of antibiotic was destroyed by the addition of 2 units of penicillinase (Riker or from *Streptomyces* R39), and the solutions were incubated at 37°C. Re-activation of the enzyme as a function of time was measured on 5 μl samples. The k_{+4} values were obtained from plots of $\ln[1-(A_t/A_0)]$ versus time where A_t is enzyme activity at time t of the incubation and A_0 is enzyme activity in the absence of antibiotic.

*Determination of the apparent rate constant (k_a) for the formation of complexes EI**

Three techniques were used, each of them having its own limitation. In all cases, the antibiotic concentration was at least 10 times higher than the enzyme concentration. Under the conditions in which the experiments were carried out, the k_a values were much higher than the k_{+4} values and therefore the simplified equation:

$$[EI^*]/E_0 = 1 - e^{-k_a t}$$

was used for the estimation of k_a (Frère *et al.*, 1975*b*).

(*a*) *Emission fluorescence of the R39 enzyme.* The fluorescence of the R61 enzyme at 320 nm (excitation, 285 nm) is decreased by 25–30% as a result of its interaction with various penicillins and by 50–60% as a result of its interaction with various cephalosporins (Frère *et al.*, 1975*b*). This particular property allowed the k_a values to be determined by using, when necessary, a stopped-flow technique. The emission fluorescence spectrum of the R39 enzyme has a maximum at 340 nm (excitation, 285 nm) (Frère *et al.*, 1974*a*). The fluorescence is not affected by penicillins, but it is decreased by 18 and 35% of the original value by reaction with cephalexin and cephalosporin C respectively. Fluorescence quenching caused by cephalosporin C was followed at 20°C with the help of a Durrum–Gibson stopped-flow apparatus modified as described by Frère *et al.* (1975*b*). The signal was recorded with a digital memory recorder Pracor 570 so that the curves were directly reproduced on graph

paper. The enzyme concentration was 1 μM and the cephalosporin C concentration varied from 10 μM to 1 mM. The k_a values were obtained from plots of $\ln(F_t - F_\infty)$ versus time where F_t is fluorescence intensity at time t of the interaction and F_∞ is fluorescence intensity after complete stabilization (slope = $-k_a$).

(*b*) *Absorbance intensity of the antibiotic.* Between 320 and 600 nm, the absorption spectrum of complex EI* formed between the R39 enzyme and the chromogenic cephalosporin 87-312 is identical with that of cephalosporin 87-312 hydrolysed by penicillinase, i.e. the maximum of the absorption spectrum shifts from 386 to 482 nm (Frère *et al.*, 1974*c*). Similarly, various cephalosporins (cephalexin, cephalosporin C and cephaloglycine) had their molar extinction coefficient at 260 nm (ε) decreased, as a result of their interaction with the R39 enzyme, to an extent similar to that obtained by penicillinase action. In 0.1 M-Tris/HCl buffer, pH 7.7, containing 0.1 M-NaCl and 0.05 M-$MgCl_2$, the ε values (litre·mol^{-1}·cm^{-1}) of intact cephalexin, cephalosporin C and cephaloglycine are 9600, 7400 and 7250 respectively. After hydrolysis with penicillinase, these values decrease to 2250, 1070 and 1500 respectively. The highest variation ($\Delta\varepsilon$ = 7350 litre·mol^{-1}·cm^{-1}) was observed with cephalexin.

The above properties exhibited by cephalosporin 87-312 and by cephalexin allowed the formation of complex EI* to be followed as a function of time. These determinations were carried out at 10°C by measuring either the increased absorbance of the reaction mixture at 482 nm (for cephalosporin 87–312) or the decreased absorbance of the reaction mixture at 260 nm (for cephalexin) with the help of the same stopped-flow apparatus as that used for fluorescence measurements. With cephalosporin 87-312, two enzyme concentrations (0.94 and 2.82 μM) were used and the antibiotic concentrations ranged from 8.7 to 175.5 μM. With cephalexin, the enzyme concentration was 2.82 μM and the antibiotic concentrations ranged from 30 to 50 μM. The k_a values were obtained from plots of $\ln \pm (E_t - E_\infty)$ versus time where E_t is absorbance at time t and E_∞ is absorbance after complete stabilization (slope = $-k_a$). The sign − or + depends on the antibiotic used, either cephalosporin 87-312 or cephalexin.

The present technique has two limitations. Accurate measurements can only be obtained with absorbance values of less than 1 (hence high antibiotic concentrations cannot be used) and at wavelengths greater than 250 nm (hence the antibiotics of the penicillin group, of which the absorbance at wavelengths greater than 250 nm is not substantially modified by reaction with the R39 enzyme, could not be studied).

(*c*) *Residual enzyme activity.* Three enzyme concentrations (1, 5 and 25 nM) were used. The following antibiotic concentrations were used: cephalosporin 87-312 (10–40 nM), benzylpenicillin (20–400 nM), carbenicillin and cephaloglycine (50–200 nM), ampicillin (50–250 nM), cephalosporin C (100–500 nM), methicillin (200–1000 nM) and cephalexin (300–1500 nM). Enzyme and antibiotic were incubated together at various temperatures (0°, 10°, 20° and 37°C). After increasing times, samples were removed, Ac_2-L-Lys-D-Ala-D-Ala (3 mM) and a β-lactamase (in amount sufficient to destroy instantaneously the excess of antibiotic) were added, and the reaction mixtures were incubated at 37°C for 1, 5 or 25 h (depending on the concentration of active enzyme). Free D-alanine was then measured under conditions of initial velocity (less than 20% of substrate was hydrolysed). As the complexed EI* exhibit very long half-lives (Table 1), their breakdown during the incubation in the presence of substrate was negligible and therefore did not affect the measurement of the residual enzyme activity. In fact, the incubation time represented at the most 15% of the half-life of complex EI*. The k_a values were obtained by plotting $\ln A_t/A_0$ versus time where A_t is residual enzyme activity at time t and A_0 is enzyme activity without antibiotic. The limitation of the technique is that very rapid reactions cannot be followed accurately and hence high antibiotic concentrations cannot be used.

Kinetic data and the determination of constants

From the plotted data, all the lines were drawn according to the best fit from least-squares regression analyses ($P < 0.001$).

Results

*First-order rate constants (k_{+4}) for the breakdown of complexes EI**

The values (at 37°C) of the constants k_{+4} for the various antibiotics tested are given in Table 1.

Interaction between the R39 enzyme and cephalosporin C as revealed by fluorescence quenching

(*a*) *Titration of the enzyme.* Samples (10 μl) containing 0.5 nmol of cephalosporin C were added, at room temperature (22°C), to 2 ml of a solution containing 3.92 nmol of R39 enzyme (the reaction was performed in 0.1 M-Tris/HCl buffer, pH 7.7, containing 0.1 M-NaCl and 0.05 M-$MgCl_2$). After each addition, the mixture was left for 10 min at room temperature, after which time the fluorescence intensity of the solution was measured at 340 nm and the enzyme activity was measured (on 10 μl samples). As shown in Fig. 1, the end point of the titration was independent of the technique used and occurred at a molar ratio of cephalosporin C/enzyme of 1.06:1. Hence, the fluorescence quenching of the R39 enzyme directly reflected its inactivation.

Table 1. *Values of the constants for the interaction between the R39 enzyme and β-lactam antibiotics*

Antibiotic	Method used	$\frac{k_{+3}}{K}$ ($M^{-1} \cdot s^{-1}$) (at the indicated temperature)	k_{+4} at 37°C (s^{-1})	Half-life of complex EI* at 37°C (min)
Methicillin	Enzyme activity	1100 (20°C)	21×10^{-6}	545
Carbenicillin	Enzyme activity	486 (0°C) 780 (10°C) 2920 (20°C) 5750 (37°C)	5.4×10^{-6}	2125
Cephalexin	Absorbance (260nm) Enzyme activity	2220 (10°C) 3000 (20°C)	2.4×10^{-6}	4800
Cephalosporin C	Fluorescence quenching Enzyme activity	67000* (20°C) 35400 (20°C)	0.28×10^{-6}	40000
Ampicillin	Enzyme activity	74000 (20°C)	4.4×10^{-6}	2600
Cephaloglycine	Enzyme activity	74000 (20°C)	0.8×10^{-6}	14000
Benzylpenicillin	Enzyme activity	13000 (0°C) >90000 (20°C) >300000 (37°C)	2.81×10^{-6}	4100
Cephalosporin 87-312	Absorbance (482nm) Enzyme activity	2280000 (10°C) ≃3000000 (0°C)	1.5×10^{-6}	7700

* $k_{+3} = 12.5 s^{-1}$; $K = 0.190$ mM.

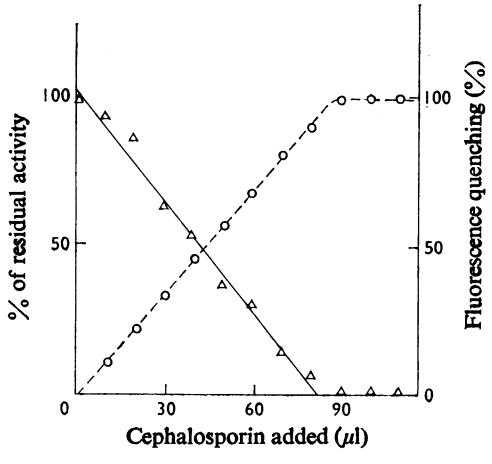

Fig. 1. *Titration of the exocellular* DD-*carboxypeptidase–transpeptidase of Streptomyces R39 with cephalosporin C*

△, On the basis of residual enzyme activity; ○, on the basis of fluorescence quenching at 340nm. For conditions, see the text.

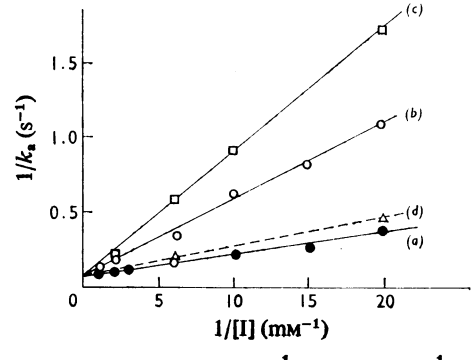

Fig. 2. *Double-reciprocal plot of* $\frac{1}{k_a}$ *versus* $\frac{1}{[\text{cephalosporin C}]}$ *at cephalosporin C concentration higher than* 50 μM

(*a*) in the absence of substrate Ac_2-L-Lys-D-Ala D-Ala; (*b*) and (*c*) in the presence of 1 mM (*b*) and 2 mM (*c*) substrate Ac_2-L-Lys-D-Ala-D-Ala; (*d*) in the absence of Ac_2-L-Lys-D-Ala-D-Ala and in the presence of 10 mM-D-alanine. For conditions, see the text.

(*b*) *Plots of* k_a *versus* [*I*] *and of* $1/k_a$ *versus* $1/[I]$. Under the conditions described in the Materials and Methods section, the plots k_a versus [I] deviated from linearity at cephalosporin C concentrations higher than 100 μM. Hence [see theoretical background in Frère *et al.* (1975*b*)], the R39 enzyme and cephalosporin C react together to form an intermediate enzyme–antibiotic complex (EI), which isomerizes (into EI*) before breakdown. From the double-reciprocal plots (Fig. 2, line *a*), a k_{+3} value of $12.5 s^{-1}$, a ratio k_{+3}/K of $67000 M^{-1} \cdot s^{-1}$ and hence a K value of 0.19 mM were calculated.

(*c*) *Plots of* $ln(1-[EI^*]/E_0)$ *versus time.* The plots obtained at a [I] value of 0.475 mM (i.e. at an inhibitor

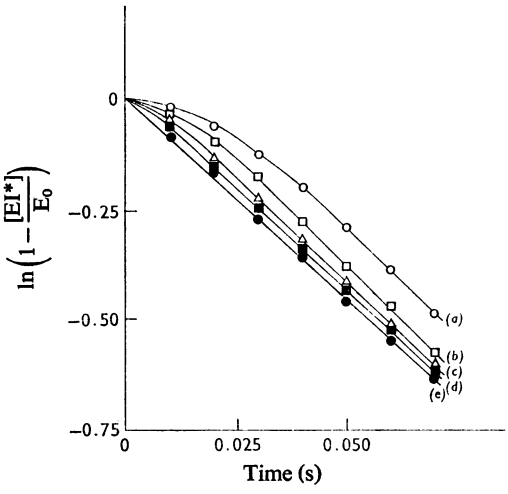

Fig. 3. *Experimental and theoretical plots* $ln(1-[EI^*]/E_0)$ *versus time for cephalosporin C*

Line (*e*), experimental plot ([E] = 1 μM; [I] = 0.475 mM). Curves (*a*), (*b*), (*c*) and (*d*), theoretical plots: (*a*) according to model $E+I \xrightarrow{k_{+1}} EI \xrightarrow{k_{+3}} EI^*$ with $k_{+1} = 67000$ $M^{-1}\cdot s^{-1}$ and $k_{+3} = 12.5 s^{-1}$; (*b*, *c*, *d*) according to model $E+I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI \xrightarrow{k_{+3}} EI^*$ with $k_{+3} = 12.5 s^{-1}$, $k_{-1} = 12.5 s^{-1}$ and $k_{+1} = 1.34\times10^5 M^{-1}\cdot s^{-1}$ (curve *b*); with $k_{+3} = 12.5 s^{-1}$, $k_{-1} = 37.5 s^{-1}$ and $k_{+1} = 2.68\times10^5 M^{-1}\cdot s^{-1}$ (curve *c*); and with $k_{+3} = 12.5 s^{-1}$, $k_{-1} = 75 s^{-1}$ and $k_{+1} = 4.7\times10^5 M^{-1}\cdot s^{-1}$ (curve *d*). The k_{+1} and k_{-1} values are such that $\frac{k_{+1}k_{+3}}{k_{-1}+k_{+3}} = 67000 M^{-1}\cdot s^{-1}$. With the highest k_{+1} and k_{-1} values (curve *d*) and within the limits of experimental error, the theoretical curve (*d*) is indistinguishable from the experimental line (*e*). ↔, Mixing dead-time (2.5 ms). For conditions, see the text.

concentration yielding a k_a equivalent to $0.70k_{+3}$) showed no lag phase (Fig. 3, line *e*). The velocity of the reaction was maximal at zero time. Consequently, the most likely model for the formation of complex EI* is $E+I \underset{}{\overset{K}{\rightleftharpoons}} EI \xrightarrow{k_{+3}} EI^*$ where the first step is a rapid-equilibrium process. Fig. 3 (curves *a*, *b*, *c* and *d*) also shows the plots that would be obtained with model $E+I \xrightarrow{k_{+1}} EI \xrightarrow{k_{+3}} EI^*$ (where $k_{+3} = 12.5 s^{-1}$ and $k_{+1} = 67000 M^{-1}\cdot s^{-1}$) and with model $E+I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI \xrightarrow{k_{+3}} EI^*$ (where $k_{+3} = 12.5 s^{-1}$ and k_{+1} and k_{-1} have increasing values such that

$$\frac{k_{+1}k_{+3}}{k_{-1}+k_{+3}} = 67000 M^{-1}\cdot s^{-1}).$$

This latter model would be compatible with the experimental data only under conditions where $k_{-1} \geqslant 6k_{+3}$ (i.e. $75 s^{-1}$) and $k_{+1} \geqslant 4.7\times10^5 M^{-1}\cdot s^{-1}$ (Fig. 3, line *d*).

(*d*) *Effects of donor* Ac_2-L-*Lys*-D-*Ala*-D-*Ala and acceptor* D-*alanine*. The interaction between enzyme, cephalosporin C and Ac_2-L-Lys-D-Ala-D-Ala may be of the competitive or the non-competitive type (Frère *et al.*, 1975*c*). Under certain conditions (Frère *et al.*, 1975*c*), the plots of $1/k_a$ versus 1/[I] for various values of [S] intercept the ordinate at a value equivalent to $1/k_{+3}$ if the model is competitive. If the model is non-competitive, the value of the intercept is a function of [S]. Enzyme (1 μM), cephalosporin C (from 498 to 910 μM, i.e. at concentrations close to the *K* constant value for the step $E+I \underset{}{\overset{K}{\rightleftharpoons}} EI$) and Ac_2-L-Lys-D-Ala-D-Ala (1 and 2 mM, i.e. at concentrations close to the K_m value for this substrate: see the Materials and Methods section) were incubated together at 20°C and the fluorescence quenching was measured as a function of time by the stopped-flow technique. As shown in Fig. 2, lines (*b*) and (*c*), the inhibition of the formation of complex EI* was of the competitive type. The occurrence of a non-competitive model, however, cannot be excluded with certainty. Indeed, if the ratio K/K' is $\leqslant 0.1$ (*K* is the dissociation constant for $EI \rightleftarrows E+I$ and *K'* is the dissociation constant for $ESI \rightleftarrows ES+I$), then both competitive and non-competitive models would give rise, within the limits of experimental error, to the same competitive plots. Hence, for a *K'* value $\geqslant$ 2 mM, the experimental results obtained would not be incompatible with a non-competitive model.

The same experiment as above was repeated except that the donor Ac_2-L-Lys-D-Ala-D-Ala was replaced by a high concentration of the acceptor D-alanine (10 mM). D-Alanine had no significant effect on the rate of formation of complex EI* (Fig. 2, line *d*).

Interaction between the R39 enzyme and either cephalosporin 87-312 or cephalexin as revealed by absorbance-intensity measurements

Because of the limitations of the technique (see the Materials and Methods section), the antibiotic concentrations used did not exceed 175.5 μM for cephalosporin 87-312 and 50 μM for cephalexin. Under these conditions and at 10°C, the plots of k_a versus [I] did not deviate from linearity and therefore the k_{+3} and *K* values could not be determined independently. The value of the ratio k_{+3}/K thus obtained was $2.66\times10^6 M^{-1}\cdot s^{-1}$ for cephalosporin 83-312 and $1.96\times10^3 M^{-1}\cdot s^{-1}$ for cephalexin.

Interaction between the R39 enzyme and various penicillins and cephalosporins as revealed by measurements of residual enzyme activity

Within the range of temperatures and antibiotic concentration used, the lines of the plots of k_a versus

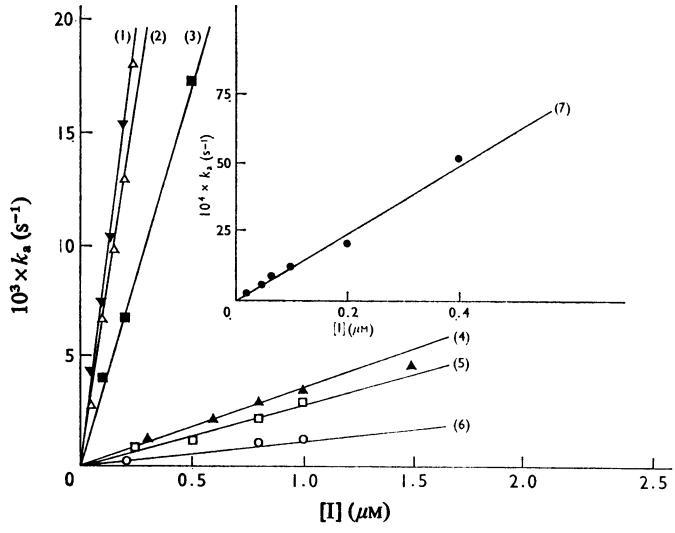

Fig. 4. *Plots of k_a versus [I] for various penicillins and cephalosporins as revealed by measurements of residual enzyme activity*

The following antibiotics were used: cephaloglycine (1), ampicillin (2), cephalosporin C (3), cephalexin (4), carbenicillin (5), methicillin (6) and benzylpenicillin (7). The experiments with benzylpenicillin were carried out at 0°C. All the others were at 20°C. For conditions, see the text.

[I] did not deviate from linearity (Fig. 4). These conditions of low antibiotic concentrations were necessary to decrease sufficiently the rates of formation of the complexes EI* so that they could be determined experimentally. The k_{+3}/K ratio values thus obtained are given in Table 1. The values for cephalosporin C, cephalosporin 87-312 and cephalexin can be compared with those obtained by physical techniques (fluorescence quenching or absorbance measurements). The various techniques gave k_{+3}/K ratio values that were in fair agreement with each other.

The antibiotics can be divided into three groups: (1) those with a low k_{+3}/K ratio value (3300–1100 $M^{-1} \cdot s^{-1}$ at 20°C) such as cephalexin, carbenicillin and methicillin (Fig. 4), (2) those with a high k_{+3}/K ratio value (90000–60000 $M^{-1} \cdot s^{-1}$ at 20°C) such as benzylpenicillin, cephaloglycine, ampicillin and cephalosporin C (Fig. 4), and (3) cephalosporin 87-312 with a k_{+3}/K ratio value of several millions $M^{-1} \cdot s^{-1}$ (even at 0°C). The influence of the temperature is important. Thus, for example, with carbenicillin and within the range 0–37°C, a 10°C increase of the temperature caused roughly a twofold increase in the k_{+3}/K value (Table 1).

In the above experiments, the k_a value for the interaction between the R39 enzyme and a given concentration of a β-lactam antibiotic was obtained from plots of $\ln A_t/A_0$ versus time (see the Materials and Methods section), hence assuming that $[EI^*]/E_0 = 1-A_t/A_0$ or $1-[E]/E_0$. The assumption is only valid if [EI] is negligible when compared with [E]. To fulfil this requirement, under our experimental conditions, the first step of the reaction must be either a rapid-equilibrium process or a slow process with $[E] \leqslant 0.2\,E_0$. Moreover, in this latter case, the measurements must be made in a range of inhibitor concentrations such that the plots k_a versus [I] show no deviation from linearity.

Discussion

On the basis of the fluorescence quenching of the R39 enzyme caused by cephalosporin C, the interaction between the enzyme and the antibiotic is a multi-step process which, qualitatively, is identical with that proposed for the interaction between the R61 enzyme and β-lactam antibiotics:

$$E+I \overset{K}{\rightleftharpoons} EI \xrightarrow{k_{+3}} EI^* \xrightarrow{k_{+4}} E+X+Y$$

(see the introduction). Although with β-lactam antibiotics other than cephalosporin C it was not possible for technical reasons to determine the K and k_{+3} values independently, it is reasonable to postulate that the above conclusions also apply to the interaction between these antibiotics and the R39 enzyme. Kinetically, the peptide donor Ac_2-L-Lys-D-Ala-D-Ala behaves as a competitive inhibitor of the interaction between the R39 enzyme and cephalosporin C, but, at the present stage of our knowledge, such behaviour

remains compatible with the formation of a ternary complex ESI.

The possible existence of a ternary complex ESI in the interaction between sensitive enzymes and β-lactam antibiotics has been much discussed (Umbreit & Strominger, 1973; Blumberg & Strominger, 1974; Frère *et al.*, 1975*c*). It appears, however, that there is no compelling evidence either for the formation of the ternary complex or for its absence. In most cases, residual enzymic activity has been measured at $[I] \ll K$, and it has been shown that competitive Lineweaver–Burk plots obtained under these conditions are not necessarily indicative of a true competitive behaviour of the enzyme–substrate–inhibitor system. Moreover, other results (Barnett, 1973; Oppenheim *et al.*, 1974; Martin *et al.*, 1975) are best explained on the basis of a non-competitive model.

Quantitatively, the R39 enzyme and the R61 enzyme differ from each other with respect to their response to β-lactam antibiotics. By comparing the data of Table 1 with those published for the R61 enzyme (Frère *et al.*, 1975*b*), it appears that: (1) in all cases, the k_{+3}/K ratio values are always higher or much higher with the R39 enzyme than with the R61 enzyme; (2) the k_{+4} values are always lower or much lower with the R39 enzyme than with the R61 enzyme; (3) the order, according to which the antibiotics can be classified on the basis of increasing k_{+3}/K ratio values or on the basis of decreasing k_{+4} values, is entirely different for each enzyme. Thus the relation between structure and activity of the β-lactam antibiotics is likely to differ greatly depending on the enzymes and the bacterial species.

The higher the k_{+3}/K ratio value and the lower the k_{+4} value, the more active is the antibiotic. Hence, alterations in the structure and/or the conformation of the transpeptidases that alter the relevant K, k_{+3} or k_{+4} values may lead to 'intrinsic' resistance. In fact, the present studies show that the R61 enzyme is either more or much more resistant than the R39 enzyme to all the antibiotics tested. In previous studies (Dusart *et al.*, 1973), ID_{50} values (i.e. the antibiotic concentrations required to inhibit by 50% the activity of the R61 and R39 enzymes in the presence of a low concentration of tripeptide donor) had been used arbitrarily to estimate the relative antibiotic sensitivity of these enzymes. If, under the conditions where they were obtained, these ID_{50} values were totally independent of the k_{+4} values (this latter parameter becomes important only at very low concentrations of antibiotic), then they should be directly related to the reciprocals of the k_{+3}/K values. By selecting for the comparison those antibiotics for which both ID_{50} values and k_{+3}/K values are known, it appears that: (1) the

$$\frac{k_{+3}/K \text{ (for the R39 enzyme)}}{k_{+3}/K \text{ (for the R61 enzyme)}}$$

ratio values (i.e., A) are 3.6, 7.3, 31, 626 and 3523 with carbenicillin, benzylpenicillin, cephalosporin C, ampicillin and cephaloglycine respectively; (2) the corresponding

$$\frac{ID_{50} \text{ (for the R61 enzyme)}}{ID_{50} \text{ (for the R39 enzyme)}}$$

ratio values (i.e. B) are 4, 2.8, 9, 867 and 1150; and therefore (3) the corresponding A/B ratio values are 0.9, 2.6, 3.4, 0.72 and 3.1. Such a correlation provides strong evidence that the model proposed for the interaction between β-lactam antibiotics and both R39 and R61 enzymes is valid and that the values experimentally determined for the constants involved in the interaction are correct.

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