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Kinetics of Interaction between the Exocellular DD-Carboxypeptidase-Transpeptidase from *Streptomyces* R61 and β-Lactam Antibiotics

A Choice of Models

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The simplest model for the interaction between the exocellular DD-carboxypeptidase-transpeptidase from *Streptomyces* R61 and β-lactam antibiotics involves the three following steps: (a) the formation of a reversible equimolar enzyme · antibiotic complex; (b) the irreversible transformation of this complex into a modified enzyme · antibiotic complex; and (c) the breakdown of this latter complex and the concomitant release of a regenerated enzyme and a modified antibiotic molecule. The dissociation constant for step 1 and the rate constants for steps 2 and 3 were measured with various β-lactam antibiotics. With an antibiotic such as benzylpenicillin, which behaves as a good 'substrate', steps 1 and 2 occur at enzymic velocities, whereas step 3 occurs at a very low velocity and hence is responsible for the low efficiency of the overall process.

The exocellular DD-carboxypeptidase-transpeptidase excreted by *Streptomyces* R61 is believed to be a solubilised form of the membrane-bound transpeptidase [1] which catalyses peptide crosslinking during the biosynthesis of the bacterial wall peptidoglycan. This exocellular enzyme has been purified to protein homogeneity [2]. It reacts with penicillins and cephalosporins to form equimolar and inactive enzyme · antibiotic complexes in which both reactants are modified [3]. Once formed, these complexes undergo spontaneous breakdown during which the enzyme is reactivated, whereas the chemically modified antibiotic is released in an inactive form [3]. The half-lives of the modified enzyme · antibiotic complexes vary depending upon the antibiotics. That of the complex formed with benzylpenicillin is about 80 min at 37 °C and the corresponding first-order rate constant for its breakdown is 1.4×10^{-4} s^{-1}. Within the limits of the methods used, only one product is released from the R61 enzyme · benzylpenicillin complex. It is neither benzylpenicillin nor benzylpenicilloic acid. Moreover, the presence of large amounts of β-lactamase during breakdown of the complex does not affect the rate constant of the reaction and does not cause the appearance of benzylpenicilloic acid [3]. On the basis of these observations, several models for the interaction between the R61 enzyme and the β-lactam antibiotics are possible. The present paper describes experiments which were carried out in order to make a choice among these models and to determine the values of the various constants involved. These studies are based upon the fact that the fluorescence of the modified 'R61 enzyme · antibiotic' complexes is lower than that of the free enzyme [4]. The approach was to use this latter property as a means of measuring the effects exerted by the antibiotic on the apparent rate constant of the formation of the 'modified R61 enzyme · antibiotic' complex and, from this, to determine which model(s) fit(s) best the observed effects.

Enzyme. β-Lactamase (penicillinase) (EC 3.5.2.6).

MODELS AND MATHEMATICAL TREATMENTS

Symbols

E = active enzyme; E_0 = concentration of total enzyme (active and inhibited); I = intact inhibitor (*i.e.* the β-lactam antibiotic); [I] = concentration of inhibitor; EI = inactive enzyme · inhibitor complex; EI* = modified enzyme · inhibitor complex; X = released and chemically modified inhibitor; k = rate constant for each of the individual steps of the reaction; K = dissociation constant; k_a = apparent rate constant for the formation of complex EI*.

Models

Two main types of models were envisaged. In type I the R61 enzyme and the inhibitor (*i.e.* penicillin) react together to form a complex EI*, in which both constituents are modified and which undergoes breakdown into E + X.

Model I (A):

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI^* \xrightarrow{k_4} E + X.$$

In type II the R61 enzyme and the inhibitor react together to form an inactive complex EI, which undergoes isomerization into EI* and finally breakdown.

Model II (A):

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_3} EI^* \xrightarrow{k_4} E + X.$$

These two models are general. Two limiting cases can be envisaged for each of them: (a) k_2 is much smaller than k_4 in type I and k_3 in type II and hence is assumed to be equal to zero.

Model I (B):

$$E + I \xrightarrow{k_1} EI^* \xrightarrow{k_4} E + X.$$

Model II (B):

$$E + I \xrightarrow{k_1} EI \xrightarrow{k_3} EI^* \xrightarrow{k_4} E + X.$$

b) The first step of the reaction is a rapid equilibrium process with $K = k_2/k_1$.

Model I (C):

$$E + I \overset{K}{\rightleftharpoons} EI^* \xrightarrow{k_4} E + X.$$

Model II (C):

$$E + I \overset{K}{\rightleftharpoons} EI \xrightarrow{k_3} EI^* \xrightarrow{k_4} E + X.$$

Model I (C) could be immediately discarded, since the rapid reversible breakdown of complex EI* with benzylpenicillin in the presence of β-lactamase would give rise to benzylpenicilloic acid, a conclusion which was in conflict with the observed facts.

Mathematical treatments of models similar to models I (A, B) and II (A, B and C) are given in [5–9]. In those studies, however, the main emphasis was on the appearance of the reaction product X, whereas the purpose of the present study was to follow the appearance of an intermediate of the reaction pathway, namely complex EI*. A choice among models and the estimation of the constants for the reaction can be made as described in the ensuing paragraphs only under the condition where [E] $\ll$ [I]. This condition is necessary to allow linearization of the differential equations and integration of the rate equations.

Choice of Models

A choice among models is based on the determination of the rate of formation of complex EI* both as a function of [I] and as a function of time.

Effect of [I]. With models I (A and B) the rate of formation of complex EI* increases continuously for increasing values of [I]. A distinction between these two models can be made on the basis of the amounts of X and I produced through the decay of the isolated complex EI*. According to model I (A) both intact and modified inhibitors are released and the ratio [I]/[X] is equal to the ratio k_2/k_4. According to model I (B) intact inhibitor is not released.

With models II (A, B and C) the rate of formation of complex EI* reaches a limiting value at high [I] values.

Effect of Time. A distinction between models II (A, B and C) can be made by measuring the rate of formation of complex EI* as a function of time at [I] values such that the rate of the reaction is about half its maximum value. With model II (C) the rate of formation of complex EI* is maximum at time zero. With models II (A and B) the rate is maximum only after a lag time. A further distinction between these two models requires the help of a proper curve-fitting program.

Estimation of the Constants

Two cases can be envisaged. If enzyme activity cannot be detected when the system has reached the steady state, k_4 is negligible and simplified equations can be used. If enzyme activity occurs at the steady state, k_4 cannot be neglected and more complex equations have to be used.

Simplified Equations (for $k_4 = 0$*).* With models I (A), I (B) and II (C) the rate of formation complex EI* is given by the equation

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

where $k_a = k_1$ [I] for models I (A and B) and $k_a = k_3/\{1 + (K/[I])\}$ for model II (C) [10–12]. Hence, with this latter model the double-reciprocal plot $1/k_a$ *versus* $1/[I]$ gives rise to a straight line, which intercepts the ordinate axis at the $1/k_3$ value and of which the slope is equal to K/k_3.

With models II (A and B) three alternatives must be examined.

a) If k_1 [I] $\gg k_3$, Eqn (1) is valid and $k_a = k_3$.

b) If k_1 [I] $\ll k_3$, Eqn (1) is also valid but $k_a = k_1$ [I] for model II (B) and $k_a = k_1$ [I] $k_3/(k_2 + k_3)$ for model II (A). Plots of k_a *versus* [I] at low [I] values yield straight lines extrapolating to the origin, of which the slope is equal to k_1 (model II, B) or $k_1k_3/(k_2 + k_3)$ (model II, A).

c) If k_1 [I] $\approx k_3$, equations derived from those presented by Ouellet and Laidler [6] can be used. For model II (A)

$$\frac{[EI^*]}{E_0} = 1 + \frac{k_1 k_3 [I]}{R}\left(\frac{e^{\alpha_1 t}}{\alpha_1} - \frac{e^{\alpha_2 t}}{\alpha_2}\right) \quad (2)$$

with

$$\alpha_1 = \frac{-(k_2 + k_3 + k_1 [I]) + R}{2}$$

$$\alpha_2 = \frac{-(k_2 + k_3 + k_1 [I]) - R}{2}$$

$$R = \sqrt{(k_2 + k_3 + k_1 [I])^2 - 4 k_1 k_3 [I]}$$

and for model II (B) (where $k_2 = 0$),

$$\frac{[EI^*]}{E_0} = 1 + \frac{k_1 [I] e^{-k_3 t} - k_3 e^{-k_1 [I] t}}{k_3 - k_1 [I]} \quad (3)$$

The best values for the various constants can be obtained from Eqns (2) and (3) with the help of a curve-fitting program.

With models II (A and B), however, a simple method can be used, which provides approximate values for the various constants. On the basis of Eqns (2) and (3) the plots ln $\{1 - ([EI^*]/E_0)\}$ *versus* time are curves which after a lag time, become progressively indistinguishable from straight lines (at least within the limits of experimental errors and between 20% and 80% of completion of the reaction). Although these 'lines' extrapolate above the origin on the ordinate axis, the slopes can be used as $-k_a$ values. The double-reciprocal plots $1/k_a$ *versus* $1/[I]$ also yield curves that are indistinguishable from straight lines and the extrapolation at $1/[I] = 0$ gives a good approximation of $1/k_3$. By using this k_3 value and the k_1 value obtained at low [I] values [see the alternative (b) discussed above], one can calculate the theoretical curves ln $\{1 - ([EI^*]/E_0)\}$ *versus* time on the basis of Eqn (3) and of model II (B). If the theoretical curves thus obtained exhibit longer lag times than those obtained experimentally, model II (B) can be rejected and model II (A) retained. On the basis of this latter model and of Eqn (2), theoretical curves can also be calculated by using increasing k_1 and k_2 values until both theoretical and experimental curves coincide (the ratio $k_1 k_3/(k_2 + k_3)$ obtained at low[I] values remaining constant for all values of k_1 and k_2).

Complete Equations (for k_4 *Not Negligible).* With models I (A, B) and II (C) the rate of formation of complex EI is given by the equation

$$\frac{[EI^*]}{E_0} = 1 - \frac{k' + k_a e^{-(k' + k_a)t}}{k' + k_a} \quad (4)$$

where $k' = k_2 + k_4$ for model I (A) and $k' = k_4$ for models I (B) and II (C), and where $k_a = k_1$ [I] for models I (A) and I (B) and $k_a = k_3/\{1 + (K/[I])\}$ for model II (C) (as in the case of the simplified equations).

When k_1 [I] is either $\gg$ or $\ll k_3$ in models II (A and B) Eqn (4) is valid; $k' = k_4$ and the k_a values are the same as those given above for the simplified equations. Finally, if k_1 [I] $\approx k_3$, the complete equation of Ouellet and Laidler [6] must be used and from it the various rate constants can be estimated with the help of a curve-fitting program.

MATERIALS AND METHODS

R61 DD-Carboxypeptidase-Transpeptidase

The exocellular R61 enzyme, purified to protein homogeneity, was used throughout these studies. The molecular weight of this enzyme is 38000 [2]. The enzyme activity was determined by measuring the release of the C-terminal D-alanine residue from the tripeptide N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine in the absence of acceptor (DD-carboxypeptidase activity) [2].

β-Lactamase

Riker β-lactamase was used. 1 unit hydrolyses 1 μmol benzylpenicillin/min.

Determination of Values of the Rate Constant k_4 *for the Breakdown of the Complexes EI**

The values previously obtained for benzylpenicillin, carbenicillin, ampicillin and penicillin V [3] were used in the present studies. The values for cephalosporin C and cephaloglycine were measured by using the same procedure (at 37 °C in 10 mM sodium phosphate) [3].

Measurements of Fluorescence Quenching of the R61 Enzyme upon Reaction with β-Lactam Antibiotics

The rate of formation of the EI* complex was estimated by measuring the fluorescence quenching of the R61 enzyme at 320 nm as a function of time [4]. All the experiments were carried out in 10 mM sodium phosphate buffer pH 7.0.

Measurements with Benzylpenicillin. Fluorescence quenching was followed at 25 °C with a Durrum-Gibson stopped-flow apparatus modified as follows [13]. The original monochromator was replaced by a larger-grating monochromator Jobin-Yvon HRS2. The light-source was a Xenon lamp (450 W, Osram).

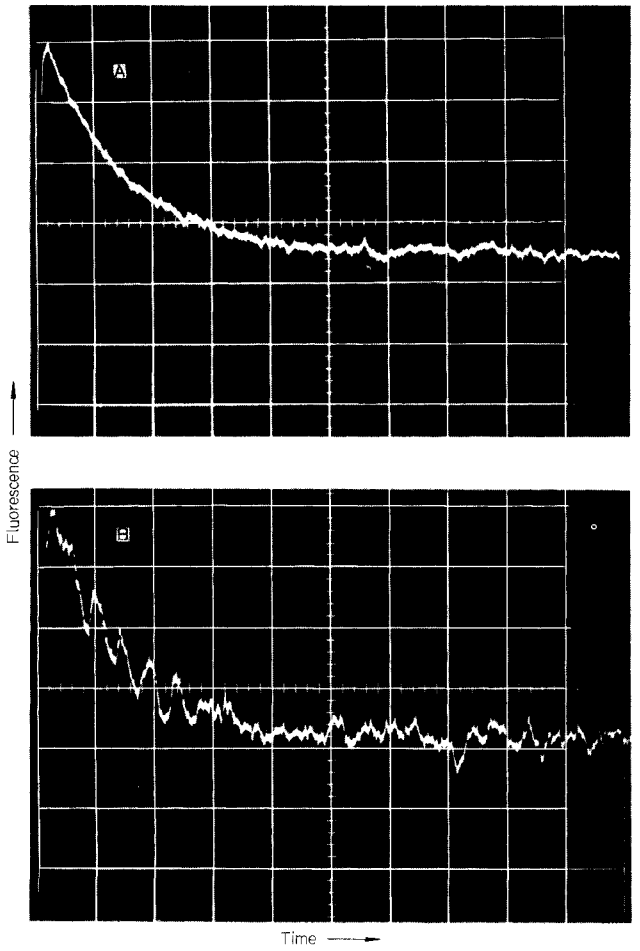

Fig. 1. *Oscilloscope tracings of the change of fluorescence of the R61 enzyme upon binding with benzylpenicillin.* Enzyme, 4.4 μM (A) and 8.8 μM (B), was mixed in the stopped-flow apparatus with 0.67 mM (A) and 25 mM (B) benzylpenicillin. The enzyme and benzylpenicillin solutions were in 10 mM sodium phosphate pH 7.0. The final concentrations were thus half the indicated values. Temperature was 25 °C. Abscissa: 200 ms/division (A) and 10 ms/division (B). The scale for fluorescence is arbitrary. Total decrease of fluorescence: 25%

The path of the excitation light (290 nm; band pass: 3 nm) was about 2 mm and the path of the emission light was 10 mm. A MTO filter (H320a) was placed in the exciting beam and a MTO filter (A340b) was placed in the path of the emission light. This latter filter transmits between 310 and 390 nm with a maximum at 330–340 nm. The fluorescence changes were amplified with a vertical amplifier 3A7 and then recorded on a memory oscilloscope (Tektronix 564R). The registered oscilloscope tracings were photographed on 36 × 24-mm films with a Nikon camera (Fig. 1) and the projected negatives were copied on graph-paper, hence giving the fluorescence changes as a function of time. In these experiments the reacting solutions (containing enzyme and benzylpenicillin respectively) were two-fold diluted (dead time: 2 ms). The concentrations, as they were expressed, referred to the final concentrations obtained after both reactants were mixed.

Measurements with Carbenicillin, Penicillin V, Ampicillin, Cephaloglycin and Cephalosporin C. The formation of the EI* complexes was followed at 37 °C with either a Baird-Atomic spectrofluorimeter (band pass: 32 nm) completed with a commercial recorder or the spectrofluorimetric set-up of M. Iwatsubo (band pass: 8 nm). In both cases the excitation wavelength was 280 nm and the emission wavelength was 320 nm.

Determination of Residual Activity at the Steady State

After stabilization of the fluorescence intensity of the reaction mixtures, 20-μl samples were removed and incubated for 20 min at 37 °C with 1 unit of β-lactamase and 100 nmol of Ac_2-L-Lys-D-Ala-D-Ala in 30 μl, final volumes, of 10 mM sodium phosphate pH 7.0. The residual activity thus measured was corrected for the breakdown of the EI* complex during the 20 min of incubation at 37 °C, as previously described [3].

Determination of the Apparent Rate Constant k_a *for the Formation of Complex EI**

After interaction with benzylpenicillin, ampicillin, cephaloglycine and cephalosporin C, the residual enzyme activity was either equal to zero or at least less than 1% of the original value. Under these conditions, E was assumed to have been totally transformed into EI* and the steady-state level of [EI*] was assumed to be equal to E_0. The k_a value was then calculated from the slope of the line obtained by plotting $\ln (F_t - F_\infty)$ *versus* time where F_t was the fluorescence intensity at time t and F_∞ the fluorescence intensity after complete stabilization [graphical procedure; slope $= -k_a$; Eqn (1)].

After interaction with carbenicillin and penicillin V, the residual enzyme activity was equal to 1–5% of the original value. It either might represent the real residual activity at the steady state or it might be due to the fact that the steady state had not been reached. Since there was no way to decide which of these alternatives was the true one, Eqn (4) was used for the calculation of the k_a values with the help of a general multiparametric curve-fitting program CFT3 (available from Prof. L. Meites, Clarkson College of Technology, Postdam, N.Y.) [14]. The k_a values were also estimated by the graphical method as described above. Only small differences were found between the two procedures.

Relative Concentrations of Enzyme and Antibiotic

The minimum value for the ratio [I]/[enzyme] used in the experiments was 9 (with benzylpenicillin).

Usually, however, the [I] value was several hundred- or several thousand fold higher than the enzyme concentration. The actual values are given in Results.

RESULTS

As discussed above, a choice among the five possible models for the interaction between the R61 enzyme and β-lactam antibiotics rested upon the analyses of both plots k_a *versus* [I] and plots $\ln \{1 - ([EI^*]/E_0)\}$ *versus* time (at [I] values such as $k_a \approx 0.5\ k_3$ *i.e.* at [I] values for which non-linearity occurs in the plots k_a *versus* [I]).

Plots k_a versus *[I] with I = Benzylpenicillin*

The plots k_a *versus* [benzylpenicillin] are shown in Fig. 2A (enzyme concentration: 1.1 μM; benzylpenicillin concentration: from 9.8 μM to 98 μM), in Fig 2B (2.2 μM enzyme; from 40 μM to 980 μM benzylpenicillin) and in Fig. 2C (4.4 μM enzyme; from 1 mM to 15 mM benzylpenicillin). The lines obtained at low concentrations of benzylpenicillin (Fig. 2A and B) extrapolated to or very close to the origin. The deviation from linearity observed at concentrations of benzylpenicillin higher than 5 mM (Fig. 2C) eliminated models I (A and B). Hence, the kinetics were consistent with model II (A, B and C). Assuming that model II (C) was the true one, and on the basis of the data of Fig. 2C, the extrapolation of the plot $1/k_a$ *versus* 1/[I] at 1/[I] = 0 gave a k_3 value of 179 s^{-1} (Fig. 3). Moreover, the slope K/k_3 of the same plot gave a K value of 13 mM (Table 1).

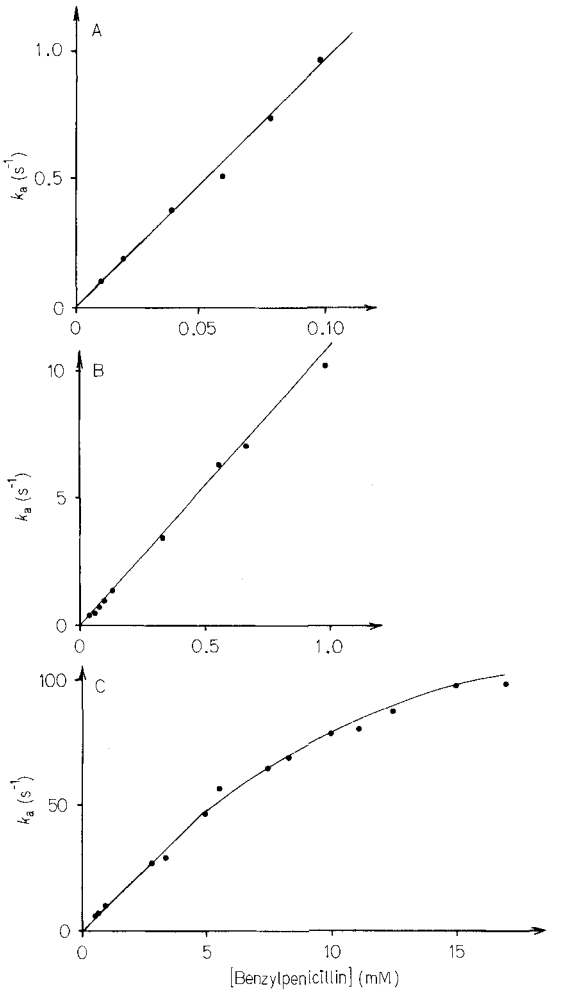

Fig. 2. *Effect of benzylpenicillin concentration on the apparent rate constant* k_a *for formation of complex EI*.* For conditions, see text. Least-square analyses of the data yield slopes of 9800 (A), and 10800 (B); average: 10300. (C) Shows deviation from linearity at benzylpenicillin concentrations higher than 5 mM

Plots k_a versus *[I] with I = Ampicillin, Penicillin V, Carbenicillin, Cephalosporin C and Cephaloglycine*

The experiments were carried out at two enzyme concentrations (0.24 μM and 0.48 μM). The following antibiotic concentrations were used: ampicillin from 0.12 mM to 5.0 mM; penicillin V: from 0.03 mM to 0.25 mM; carbenicillin: from 0.02 mM to 0.12 mM; cephalosporin C: from 0.01 mM to 0.3 mM and cephaloglycine: from 0.03 mM to 0.7 mM. The maximal fluorescence quenching caused by ampicillin, penicillin V and carbenicillin was similar to that caused by benzylpenicillin (25–30% of the original value). Cephalosporin C and cephaloglycine caused a much higher decrease of the fluorescence of the enzyme (Fig. 4). With all the antibiotics, the plots k_a *versus* [I] extrapolated to $k_a = 0$ for [I] = 0. At high concentrations of ampicillin, carbenicillin and cephaloglycine, the plots deviated from linearity. Hence, the effect caused by these antibiotics was, in all respects, similar

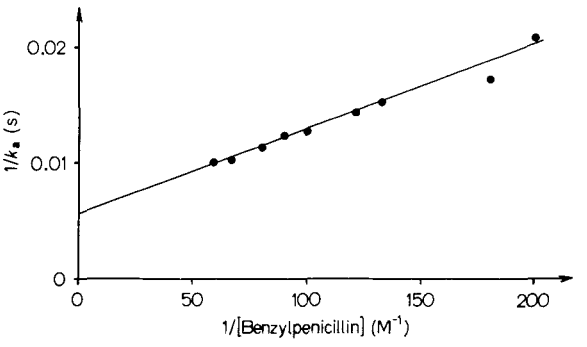

Fig. 3. *Double-reciprocal plot of* $1/k_a$ versus *1/[benzylpenicillin] at benzylpenicillin concentrations higher than 5 mM.* The experimental conditions are those of Fig. 2. Least-square analyses of the data yield an intercept value of 5.59×10^{-3} (*i.e.* $1/k_3$) and a slope of 0.73×10^{-4} (*i.e.* K/k_3), according to model II (C). The K value is thus 13 mM. The value of the ratio k_3/K (13700) is close to the 10300 value obtained from Fig. 2A and B, at low antibiotic concentrations

Table 1. *Values of the constants for model II (C)*

$E + I \overset{K}{\rightleftharpoons} EI \overset{k_3}{\rightarrow} EI^* \overset{k_4}{\rightarrow} E + X$ (at 37 °C unless otherwise indicated). With penicillin V and cephalosporin C, only minimum values could be assigned to constants K and k_3 (see text)

Antibiotic	K	k_3	k_3/K	k_4
	mM	s^{-1}	$M^{-1}\,s^{-1}$	s^{-1}
Benzylpenicillin	13 (25 °C)	179 (25 °C)	13700[a] (25 °C)	0.21×10^{-4} (25 °C)
			10300 (25 °C)	1.4×10^{-4}
Carbenicillin	0.109	0.091	830	1.4×10^{-4}
Ampicillin	7.2	0.77	107	1.4×10^{-4}
Penicillin V	>1	>1	1500	2.8×10^{-4}
Cephalosporin C	>1	>1	1150	1×10^{-6}
Cephaloglycine	0.4	0.0085	21	3×10^{-6}

[a] The greater value was obtained from the slope of $1/k_a$ *versus* $1/[I]$ at high [I]. The smaller value was obtained from the slope of k_a *versus* [I] at low [I].

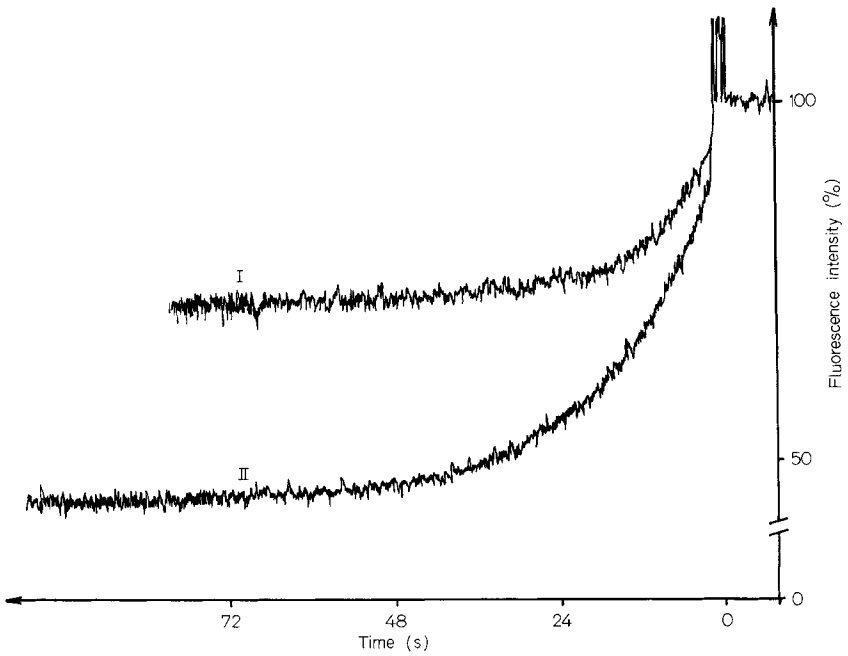

Fig. 4. *Change of fluorescence of 0.24 μM R61 enzyme upon binding (I) with 62.5 μM penicillin V and (II) with 56.4 μM cephalosporin C.* Buffer: 10 mM sodium phosphate pH 7.0. Temperature: 37 °C. The mentioned concentrations are those before the reactants were mixed. Spurious tracings on the left side of time-zero were due to the mixing of the reactants. The experiments were performed with the set-up of M. Iwatsubo

to that caused by benzylpenicillin and the kinetics were consistent with models II (A, B and C). Table 1 gives the K and k_3 values for model II (C).

With penicillin V and cephalosporin C, deviation from linearity could not be observed with certainty at the highest concentrations of antibiotic used. Unfortunately, at concentrations higher than those, the reactions were too rapid to be followed with accuracy. Hence, only the k_3/K values for model II (C) could be calculated (Table 1).

With carbenicillin, the k_a value was estimated both by the graphical method and by the use of the computer. No important differences were observed between the two methods (Table 2).

Plots of ln $\{1 - ([EI^*]/E_0)\}$ versus *Time*

In order to be useful as a means of making a choice between models II(A, B and C) the plots $\ln \{1 - ([EI^*]/E_0)\}$ *versus* time have to be analyzed at a range of inhibitor concentrations which causes non-linearity in the plots k_a *versus* [I]. As shown above, the experiments carried out with penicillin V and cephalosporin C did not fulfil this requirement.

The plots obtained at concentrations of cephaloglycine yielding k_a values from $0.45 \times k_3$ to $0.70 \times k_3$ showed no lag time. Moreover, Fig. 5 compares the experimental plot (curve 1) obtained at 5.8 mM cephaloglycine and the theoretical one (curve 5) con-

Table 2. *Values of* k_a, k_3 *and* K *relative to model II (C), obtained from simplified and complete equations*
K and k_3 values were obtained from plots $1/k_a$ *versus* 1/[carbenicillin]. When the simple method [Eqn (2)] was used, only the values obtained at $[EI^*]/E_0 \leq 0.80$ were considered. The better correlation coefficient obtained by using the simple method was probably due to the fact that an error on the estimation of time-zero influenced the computation with the complete equation but not the computation with the simple method. Hence, the values obtained with the simple equation were probably more reliable

Constant	Carbenicillin concentration	Values of constants from	
		simplified Eqn (2)	complete Eqn (1)
	μM	s^{-1}	
k_a	19.8	0.0146	0.0118
	24.7	0.0168	0.0133
	29.0	0.0185	0.0171
	29.7	0.0192	0.0132
	37.0	0.0224	0.0175
	49.5	0.0252	0.0211
	58.0	0.0330	0.0301
	87.0	0.045	0.042
	116	0.050	0.046
k_3		0.091	0.106
K		1.09×10^{-4} M	1.66×10^{-4} M
k_3/K		830 M^{-1} s^{-1}	640 M^{-1} s^{-1}
Correlation coefficient		0.99	0.98

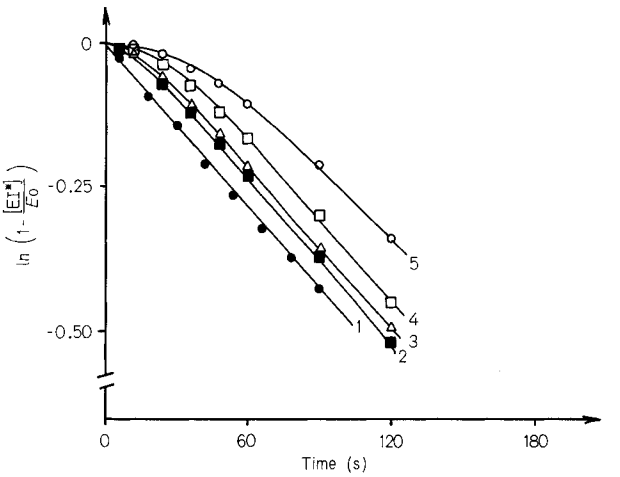

Fig. 5. *Experimental and theoretical plots ln* $\{1 - ([EI^*]/E_0)\}$ versus *time for cephaloglycine.* Curve (1): experimental plot. [I] = 5.8 mM; [E] = 0.24 μM. Curves 2, 3, 4 and 5: theoretical plots. According to model II (B): curve 5; $k_2 = 0$; $k_1 = 21$ M^{-1} s^{-1}; $k_3 = 8.5\times10^{-3}$ s^{-1}. Theoretical plots according to model II (A) and for a k_3 value of 8.5×10^{-3} s^{-1}. Curve 4: $k_1 = 42$ M^{-1} s^{-1}; $k_2 = k_3 = 8.5\times10^{-3}$ s^{-1}. Curve 3: $k_1 = 84$ M^{-1} s^{-1}; $k_2 = 3\times k_3 = 25.5\times10^{-3}$ s^{-1}. Curve 2: $k_1 = 126$ M^{-1} s^{-1}; $k_2 = 5\times k_3 = 42.5\times10^{-3}$ s^{-1}. The k_1 values for model II (A) were obtained by multiplying the k_1 values used when $k_2 = 0$, by the factor $(k_2 + k_3)/k_3$ (see theoretical section)

Table 3. *Values of the constants* k_1, k_2 *and* k_3 *relative to model II (A) (at 37 °C unless otherwise stated)*
Note that $(k_2 + k_3)/k_1 \approx K$

Antibiotic	Min k_1	Min k_2	Approx. k_3
	M^{-1} s^{-1}	s^{-1}	
Benzylpenicillin (25 °C)	2×10^4	179	179
Carbenicillin	1.66×10^3	0.09	0.087
Ampicillin	1.8×10^2	0.5	0.77
Cephaloglycine	1.2×10^2	0.04	0.0085

structed on the basis of model II (B) by using a k_2 value of zero, the k_3 value of 8.5×10^{-3} s^{-1} (Table 1) and a k_1 value of 21 M^{-1} s^{-1} (*i.e.* a k_1 value equivalent to k_3/K; Table 1). Obviously, model II (B) can be eliminated. Model II (A), however, remained possible. To illustrate this point, Fig. 5 also shows the theoretical plots (curves 2, 3 and 4) constructed on the basis of model II (A) by using the k_3 value of 8.5×10^{-3} s^{-1}, various values of k_2 ranging from $1\times k_3$ to $5\times k_3$ and the corresponding values of k_1 (see legend of Fig. 5). With $k_2 = 5\times k_3$, the theoretical curve and the experimental line virtually parallel each other and are practically superimposable, at least within a limit of time of about 5–6 s, a value which is very close to the 'dead time' required to mix both reactants (2–3 s).

The plots ln $\{1 - ([EI^*]/E_0)\}$ *versus* time obtained with ampicillin and carbenicillin also showed no lag time. Since, however, the reaction with these two antibiotics was still faster than that with cephaloglycine, the 'mixing dead time' might have been too long and hence a lag time might have escaped detection.

The experiments with benzylpenicillin were performed with the help of a stopped-flow apparatus and the mixing dead time was decreased to 2 ms. Once more, no lag time was observed in the plots of ln $\{1 - ([EI^*]/E_0)\}$ *versus* time. Fig. 1 shows the oscilloscope tracing obtained in the presence of 12.5 mM benzylpenicillin (final concentration) under which conditions the k_a value was 88 s^{-1}, *i.e.* equivalent to 0.5× the k_3 value. Within the limits of experimental error, however, a very short lag time of 1 ms might occur. This latter value, when added to the mixing dead time, gave a maximum lag time of 3 ms from which a minimum value of 179 s^{-1} for k_2 in model II (A) could be estimated.

On the basis of model II (A), Table 3 gives the approximate values of k_3 and the minimum values of k_1 and k_2 for the various β-lactam antibiotics studied. Note that $(k_3 + k_2)/k_1$ is roughly equal to the constant K of model II (C).

DISCUSSION

The interaction between the R61 enzyme and the β-lactam antibiotics proceeds most likely according to model II (C). Model II (A), however, cannot be eliminated. A choice between these two mechanisms could be made if, at a given time of the interaction when complex EI represented a high proportion of the total enzyme involved in the reaction, it were possible to destroy instantaneously all the excess of antibiotic (for example with β-lactamase). With model II (C) the EI complex would instantaneously dissociate into E and the β-lactamase–hydrolyzed antibiotic, and, parallel to this, the decrease of fluorescence intensity would immediately stop. With model II (A) only the fraction $k_2/(k_3 + k_2)$ of the complex EI formed would revert to E + the β-lactamase–hydrolyzed antibiotic and, therefore, further quenching of the fluorescence would occur. In practice such an experiment would be either exceedingly difficult or even impossible to perform (as, for example, in the case of cephaloglycine for which $k_2 \gg k_3$ on the basis of model II (A). The same steps are involved in both models II (C and A), which therefore are not essentially different. Model II (C), however, is the simplest one that accounts for all the experimental facts so far accumulated. Until further progress can be made, it should be considered as the one which best represents the mechanism of the reaction.

The breakdown of the EI* complexes gives rise to the regenerated enzyme and to a modified antibiotic. Hence, the R61 enzyme does catalyse the reaction I → X, although with a very low efficiency. With benzylpenicillin, the k_{cat} value for this reaction is 1.4×10^{-4} s^{-1}. However, the first-order kinetic constant k_3 for the isomerization EI → EI* is 180 s^{-1}. This latter value is similar to the k_{cat} value of a true enzymic reaction, for example, the hydrolysis by the same R61 enzyme of a good substrate (about 50 s^{-1}). It thus follows that the isomerization EI → EI* during the interaction between the R61 enzyme and benzylpenicillin, occurs at an 'enzymic velocity' whereas the dissociation EI* → E + X occurs at a low non-enzymic velocity. In fact, Table 1 shows the specificity profile of the R61 enzyme for various antibiotics considered as substrates. It is worth mentioning that, on the basis of model II (C) the K values varied to a much lesser extent than the k_3 values, depending upon the antibiotics (Table 1). At the most, the K values exhibited a 120-fold variation (between benzylpenicillin and carbenicillin) and the k_3 values a 20000-fold variation (between benzylpenicillin and cephaloglycine). Note also that there was only a 300-fold variation between the highest and lowest k_4 values (penicillin V and cephalosporin C).

At low antibiotic concentrations, the important parameter for activity is not the K value alone as previously suggested by Umbreit and Strominger [15] but is the ratio k_3/K in model II (C) and the ratio $k_1k_3/(k_2 + k_3)$ in model II (A). At very low concentrations, however, the activity of the inhibitor is also much dependent upon the k_4 value. Indeed, at the steady state $[EI^*]/E_0 = 1 - [k_4/(k_4 + k_a)]$ and hence, the higher the k_4 value, the smaller is that part of the total enzyme which is immobilized in the form of complex EI*. The value of $[E]/E_0$ at the steady state can be rigorously estimated:

$$\left(\frac{[E]}{E_0}\right)_{\text{steady state}} = \frac{1}{1 + \frac{[I]}{K}\left(1 + \frac{k_3}{k_4}\right)} \quad \text{(model II, C)}$$

$$\left(\frac{[E]}{E_0}\right)_{\text{steady state}} = \frac{k_2 + k_3}{k_2 + k_3 + k_1[I]\left(1 + \frac{k_3}{k_4}\right)} \quad \text{(model II, A).}$$

Increased k_4 values necessarily cause increased steady-state levels of active enzyme E or, in other words, the higher the k_4 value the more resistant is the enzyme to penicillin inhibition, even if the values of the constants K and k_3 in model II (C) or of the corresponding constants in model II (A) remain unchanged. This suggests a possible mechanism of resistance to penicillin in addition to those which were already discussed [16]. It is worth mentioning that with benzylpenicillin the k_4 value for the DD-carboxypeptidase of *Bacillus stearothermophilus* is about 1×10^{-3} s^{-1} (at 55 °C) [17] and that the k_4 value for the exocellular DD-carboxypeptidase-transpeptidase of *Streptomyces* R39 is about 3×10^{-6} s^{-1} (at 37 °C) [18]. These values are about 10 times higher and 100 times lower, respectively, than that of the R61 enzyme for the same antibiotic (at 37 °C).

The data presented here are in fair agreement with those previously published by Nieto *et al.* [4], although the interpretation proposed at that time for the interaction between the R61 enzyme and benzylpenicillin (an equilibrium process: $E + I \underset{k_r}{\overset{k_f}{\rightleftharpoons}} EI$, with $K_a = k_f/k_r$) must be modified. That interpretation was based on experiments carried out at low and equimolar concentrations of antibiotic and enzyme and the k_f constant was presented as a second-order rate constant. It can be easily demonstrated that under these conditions the formation of complex EI*, according to models II (C and A), follows second-order kinetics. Consequently, the former k_f constant corresponds either to k_3/K (model II, C) or to $k_1k_3/(k_2 + k_3)$ (model II, A). Similarly, the former k_r constant corresponds to k_4 and the former association constant K_a corresponds to the ratio k_3/k_4K (model II, C) or

the ratio $[k_1/k_4]\,[k_3/(k_2 + k_3)]$ (model II, A). The experimental values are in very good agreement: $k_f = 1 \times 10^4$; k_3/K or $k_1k_3/(k_2 + k_3) = 1.03 \times 10^4$ $M^{-1}\ s^{-1}$; $k_r = 1 \times 10^{-4}\ s^{-1}$; $k_4 = 0.2 \times 10^{-4}\ s^{-1}$.

In previous studies [3] benzylpenicillin, penicillin V and carbenicillin had been shown to behave kinetically as competitive inhibitors of the R61 enzyme with regard to the hydrolysis of the peptide donor. The antibiotic concentrations used in these experiments, however, were always much smaller than the dissociation constant K values (Table 1) for the corresponding antibiotics. Hence, the observed inhibition was actually due to the immobilization of the enzyme in the form of complex EI*. The interpretation of these results is given in the next paper of this series [19].

Finally, the fact that the quenching of the fluorescence of the R61 enzyme caused by cephaloglycine and cephalosporin C was much more intense than that caused by the other β-lactam antibiotics is worth mentioning. Parallel to this, the complexes EI* formed with these two cephalosporins were about 100-fold more stable than the complexes formed with the other antibiotics. These two phenomena might be related to each other.

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