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

A Choice of Models

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The interaction between the exocellular DD-carboxypeptidase-transpeptidase of *Streptomyces* R61 and β-lactam antibiotics is a multistep process during which a rather stable enzyme · antibiotic complex is formed. This mechanism of interaction is compatible with Lineweaver-Burk plots that are typical of a competitive inhibition of the hydrolysis of the peptide donor by the antibiotic. In fact, however, the same Lineweaver-Burk plots can be obtained on the basis of a non-competitive type of inhibition. At present, a choice between the two models cannot be made.

Recent studies showed that the interaction between the exocellular DD-carboxypeptidase-transpeptidase from *Streptomyces* R61 (E) and β-lactam antibiotics (I) is a multistep reaction [1]. Once formed, the equimolar and inactive complexes EI give rise to modified complexes EI* which, in turn, undergo breakdown with the concomitant release of the reactivated enzyme (E) and of a modified and inactive antibiotic molecule (X). Depending upon the antibiotic, the EI* complexes have at 37 °C long half-lives of 40–11 000 min. The rates of formation of complexes EI*, as determined by measuring the quenching of the fluorescence of the R61 enzyme in the presence of large amounts of antibiotics, suggested that the simplest mechanism which accounted for all the experimental results so far accumulated, was:

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

where the formation of complex EI was a rapid equilibrium process [1].

Earlier studies [2] had shown that by incubating together the R61 enzyme, the tripeptide Ac_2-L-Lys-D-Ala-D-Ala donor and a β-lactam antibiotic for some time (30–60 min) at 37 °C and on the basis of the residual activity measured under these conditions, Lineweaver-Burk plots were obtained according to which the R61 enzyme was competitively inhibited by the antibiotics with regard to the peptide donor (DD-carboxypeptidase activity: Ac_2-L-Lys-D-Ala-D-Ala + H_2O → D-Ala + Ac_2-L-Lys-D-Ala). The enzyme used in these experiments had been purified to protein homogeneity; the antibiotic concentrations were much higher than that of the enzyme and the amount of hydrolysis product formed never exceeded 15% of the original amount of substrate. Because of this low utilization of substrate, it was assumed that the experiments had been carried out under conditions of initial velocity. On the basis of the model proposed above for the interaction between the enzyme and penicillin, however, it appears that this assumption was not justified. Indeed, the estimation of the velocity of the formation of complex EI* showed that the concentration of free enzyme and hence the velocity of the reaction continuously decreased throughout the incubation of the reaction mixture. It thus became essential to examine whether or not the 'competitive' inhibition (in Lineweaver-Burk plots) was compatible with the mechanisms proposed for the interaction between the R61 enzyme and penicillin.

THEORETICAL BACKGROUND

Symbols

The symbols are those used previously [1]. In addition, S = substrate (Ac_2-L-Lys-D-Ala-D-Ala); [S] = substrate concentration; P = products of the enzyme

reaction (Ac_2-L-Lys-D-Ala and D-Ala). Moreover, whereas k_a is the apparent rate constant for the formation of complex EI* in the absence of substrate [1], k'_a is the apparent rate constant for the formation of complex EI* in the presence of substrate.

Models

Theoretically the concomitant interaction between the R61 enzyme, the donor substrate and the inhibitor could be either competitive, non-competitive or uncompetitive. An uncompetitive model, however, can be immediately discarded, since β-lactam antibiotics bind to the R61 enzyme in the absence of the substrate [1]. In both competitive and non-competitive models the step ES → E + P (*i.e.* the hydrolysis of the substrate) was considered to be irreversible, since on the basis of earlier studies [3] reversion of the enzyme reaction does not occur. Similarly, product inhibition was never observed [3].

Competitive Model. A ternary complex EIS cannot be formed

$$\begin{array}{l} \quad E + I \overset{K}{\rightleftharpoons} EI \overset{k_3}{\rightarrow} EI^* \overset{k_4}{\rightarrow} E + X \\ \quad + \\ K_s \left\{ \begin{array}{l} \quad S \\ k'_1 \downarrow\uparrow k'_2 \\ \quad ES \\ \quad \downarrow k'_3 \\ E + P \end{array} \right. \end{array}$$

with

$$K_s = \frac{k'_2 + k'_3}{k'_1}.$$

Non-competitive Model. The ternary complex EIS does occur:

$$\begin{array}{lll} E + I \overset{K}{\rightleftharpoons} & EI \overset{k_3}{\rightarrow} EI^* \overset{k_4}{\rightarrow} & E + X \\ + & + & \\ S & S & \\ K_m \downarrow\uparrow & \downarrow\uparrow K'_m & \\ ES + I \overset{K'}{\rightleftharpoons} & EIS & \\ \downarrow k'_3 & & \\ E + P & & \end{array}$$

In this model the four steps of the loop are rapid equilibrium processes. Therefore $K_m K' = K K'_m$.

Mathematical Treatments

According to model II (C) [1]

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

the concentration of complex EI* at time t is given by the equation

$$\frac{[EI^*]}{E_0} = 1 - \frac{k_4 + k_a e^{-(k_4 + k_a)t}}{k_4 + k_a}.$$

Assuming that the steps where the substrate is involved are at the steady state, the concentration of EI* at time t in the presence of substrate is given by

$$\frac{[EI^*]}{E_0} = 1 \frac{k_4 + k'_a e^{-(k_4 + k'_a)t}}{k_4 + k'_a} \quad (1)$$

where

$$k'_a = \frac{k_3}{1 + \frac{K}{[I]} + \frac{K}{[I]}\frac{[S]}{K_s}} \quad (2)$$

if the model is competitive or

$$k'_a = \frac{k_3}{1 + \frac{K}{[I]} + \frac{K}{[I]}\frac{[S]}{K_m} + \frac{[S]}{K'_m}} \quad (3)$$

if the model is non-competitive. 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. On the contrary, if the model is non-competitive, the value of the intercept is a function of [S] according to the equation

$$\left(\frac{1}{k'_a}\right)_{1/[I]=0} = \frac{1}{k_3}\left(1 + \frac{[S]}{K'_m}\right).$$

As long as the amount of product formed is negligible when compared to the initial concentration of substrate, [ES] at time t is given by

$$\frac{[ES]}{E_0} = \frac{k_4 + k'_a e^{-(k_4 + k'_a)t}}{b(k_4 + k'_a)} \quad (4)$$

where k'_a has the values given above [Eqns (2) and (3)] and where

$$b = 1 + \frac{K_s}{[S]}\left(1 + \frac{[I]}{K}\right) \quad (5)$$

if the model is competitive and

$$b = 1 + \frac{K_m}{[S]}\left(1 + \frac{[I]}{K}\right) + \frac{[I]}{K'} \quad (6)$$

if the model is non-competitive.

Assuming P_M to be the amount of product that would be formed after time t_1 if all the enzyme were in the form ES ($P_M = k'_3 E_0 t_1$) and P the amount of

product formed after the same time t_1 in the presence of inhibitor and at a non-saturating concentration of substrate $\left(P = k_3' \int_0^{t_1} [\mathrm{ES}]\, \mathrm{d}t\right)$, it follows that $P/P_M = 1/t_1 \int_0^{t_1} ([\mathrm{ES}]/E_0)\, \mathrm{d}t$. By taking equation no. 4 into account and after integration

$$\frac{P}{P_M} = \frac{k_4}{b\,(k_4 + k_a')} + \frac{k_a'}{b\,(k_4 + k_a')^2\, t_1}\,[1 - e^{-(k_4 + k_a')t_1}]. \tag{7}$$

From Equation it follows that after a given time t of incubation, the plots P_M/P *versus* $1/[S]$ give rise to a series of curves exhibiting upward concavity. They either converge on the same value of the ordinate axis if the model is competitive or they intersect the ordinate axis at various values if the model is non-competitive. Two alternatives can be envisaged.

a) For large values of time, the steady-state term of Eqn (7) [*i.e.* $k_4/b\,(k_4 + k_a')$] is largely predominant. The curves become straight lines and the plots exhibit the characteristic features of 'classical' competitive and non-competitive inhibition. Indeed, when the two non-steady-state terms are negligible

$$\left(\frac{P_M}{P}\right)_{\text{steady state}} = 1 + \frac{K_s}{[S]}\left(1 + [I]\frac{k_4 + k_3}{k_4 K}\right)$$

if the model is competitive and

$$\left(\frac{P_M}{P}\right)_{\text{steady state}} = 1 + \frac{K_m}{[S]}\left(1 + [I]\frac{k_4 + k_3}{k_4 K}\right) + \frac{[I]}{K'}$$

if the model is non-competitive. Furthermore, if $[I] \ll K'$, then a non-competitive model itself yields plots that are typical of a competitive inhibition.

b) For small values of time, the steady-state term of Eqn (7) is not predominant. Even under these general conditions, however, one can show that if $[I] \ll K$, then the Eqns (2) and (3) and Eqns (5) and (6) simplify to

$$k_a' = \frac{k_3\,[I]}{K\left(1 + \dfrac{[S]}{K_s}\right)} \tag{8}$$

if the model is competitive, or to

$$k_a' = \frac{k_3}{\dfrac{K}{[I]}\left(1 + \dfrac{[S]}{K_m}\right) + \dfrac{[S]}{K_m'}} \tag{9}$$

if the model is non-competitive. And

$$b = 1 + \frac{K_s}{[S]} \tag{10}$$

if the model is competitive, or

$$b = 1 + \frac{K_m}{[S]} + \frac{[I]}{K'} \tag{11}$$

if the model is non-competitive.

Furthermore, if (in addition to $[I] \ll K$) $K \approx K'$ (and $K_m \approx K_m'$ since $K_m K' = K K_m'$), Eqn (9) is similar to Eqn (8) and Eqn (11) is similar to Eqn (10), *i.e.* product formation is identical whether the model is competitive or non-competitive.

MATERIALS AND METHODS

R61 Enzyme and Substrate

The activity of the R61 enzyme was estimated by measuring the amount of D-alanine released from the tripeptide N^{α},N^{ε}-diacetyl-L-lysyl-D-alanyl-D-alanine [4]. The Michaelis constant value (as determined from experiments carried out in the absence of antibiotic) was about 10 mM [5]. The substrate was always used at a concentration close to this value.

Antibiotics and Values of the Constants K, k_3 *and* k_4

Benzylpenicillin, carbenicillin and penicillin V were used. The values of the various constants for the interaction of each of these antibiotics with the R61 enzyme were those determined previously in the absence of substrate [1]. Unless otherwise stated, these values were determined at 37 °C. The K values were 13 mM for benzylpenicillin (at 25 °C) and 0.109 mM for carbenicillin. The k_3 values were 179 s^{-1} for benzylpenicillin (at 25 °C) and 0.091 s^{-1} for carbenicillin. The k_4 values were 1.4×10^{-4} s^{-1} for both benzylpenicillin and carbenicillin. For penicillin V, the exact K and k_3 values were not known but the ratio k_3/K was 1500 M^{-1} s^{-1} (with $k_3 > 1$ s^{-1} and $K > 1$ mM) and the k_4 value was 2.8×10^{-4} s^{-1} [1].

Determination of the Rate Constant k_a' *for the Formation of Complex EI* with Carbenicillin in the Presence of Substrate*

Substrate and enzyme, in 10 mM sodium phosphate buffer pH 7.0, were separately pre-incubated at 37 °C. They were rapidly mixed and the antibiotic was immediately added. The final concentrations of enzyme (0.24 μM) and of Ac_2-L-Lys-D-Ala-D-Ala (6.5 mM and 13 mM) were such that less than 5% of the substrate was hydrolysed in 1 min. The concentrations of carbenicillin (from 0.029 mM to 0.116 mM) were equivalent to 0.26–1.05× the K value. Quenching of the fluorescence of the enzyme upon addition of carbenicillin was measured as described previously [1] and the k_a' values were calculated from the slopes

Table 1. *Theoretical and experimental values of* P/P_M
The experimental results for benzylpenicillin were obtained at 37 °C. The values of the constants used for the theoretical P/P_M values were measured at 37 °C (k_4) and 25 °C (k_3 and K)

Antibiotic	Substrate	P/P_M for $K/[I]$ =					
		96.4		48.4		32.1	
		theor.	exp.	theor.	exp.	theor.	exp.
	mM						
Carbenicillin [2]	3.9	0.123	0.153	0.073	0.105	0.051	0.074
	5.2	0.157	0.200	0.097	0.134	0.068	0.097
	7.8	0.221	0.245	0.144	0.183	0.099	0.138
	15.5	0.365	0.434	0.250	0.292	0.185	0.222
		for $K/[I]$ =					
		8.7×10^4		5.6×10^4		4.2×10^4	
		theor.	exp.	theor.	exp.	theor.	exp.
Benzylpenicillin [5]	2.15	0.061	0.077	0.045	0.061	0.0335	0.044
	4.3	0.116	0.155	0.085	0.122	0.063	0.095
	6.6	0.172	0.243	0.126	0.170	0.100	0.135
		for K/k_3 [I] =					
		1.29×10^3		0.89×10^3		0.45×10^3	
		theor.	exp.	theor.	exp.	theor.	exp.
Penicillin V [2]	3.9	0.152	0.150	0.124	0.125	0.077	0.081
	5.2	0.194	0.190	0.160	0.155	0.101	0.094
	7.8	0.265	0.260	0.223	0.220	0.145	0.150
	15.5	0.420	0.450	0.367	0.370	0.257	0.270

of the lines obtained by plotting $\ln (F_t - F_\infty)$ *versus* time [1], at t values lower than 1 min.

Determination of P/P_M *Values after Incubation of the R61 Enzyme with Substrate and Antibiotic*

The experimental data used were those previously published [2]. In all cases less than 13% of substrate was transformed into product so that Eqn (7) could be used. With benzylpenicillin [2,5], enzyme (a crude preparation had been used; it was equivalent to a 26 nM concentration of pure enzyme), and substrate (2.15, 4.30 and 6.6 mM) were incubated for 30 min at 37 °C in 0.01 M Tris-HCl buffer pH 7.5 in the absence and in the presence of various concentrations of antibiotic (0.15, 0.23 and 0.31 μM). The $K/[I]$ values were thus equal to 4.2×10^4, 5.6×10^4 and 8.7×10^4 respectively.

With carbenicillin and penicillin V [2], enzyme (17 nM) and substrate (3.9, 5.2, 7.8 and 15.5 mM) were incubated for 60 min at 37 °C in 10 mM sodium phosphate buffer pH 7.5 in the absence and in the presence of various antibiotic concentrations [2]. The concentrations of carbenicillin were 3.4 μM, 2.25 μM and 1.13 μM, and the corresponding $K/[I]$ values were thus equal to 32.1, 48.4 and 96.4. The concentrations of penicillin V were 1.52 μM, 0.76 μM and 0.53 μM and the corresponding $K/k_3[I]$ values were 450, 890 and 1290. On the basis of a minimum value of 1 mM for the constant K [1], the minimum value for $K/[I]$ (at $[I]_{maximum}$ = 1.52 μM) was 650.

RESULTS

Comparison between Theoretical and Experimental P/P_M *Values on the Basis of the Competitive Model in the Presence of Benzylpenicillin, Carbenicillin and Penicillin V*

With each of these antibiotics, the conditions $[E] \ll [I]$ and $[I] \ll K$ were fulfilled (see Materials and Methods and Table 1). The K_s constant was given the Michaelis constant value of the enzyme found experimentally for Ac_2-L-Lys-D-Ala-D-Ala (10 mM); the K, k_3 and k_4 values were those previously measured [1] and the substrate concentrations, antibiotic concentrations and incubation times were those which had been actually used for the kinetics previously published [2,5]. The k'_a values were calculated from Eqn (8), and the b values from Eqn (10). It thus follow-

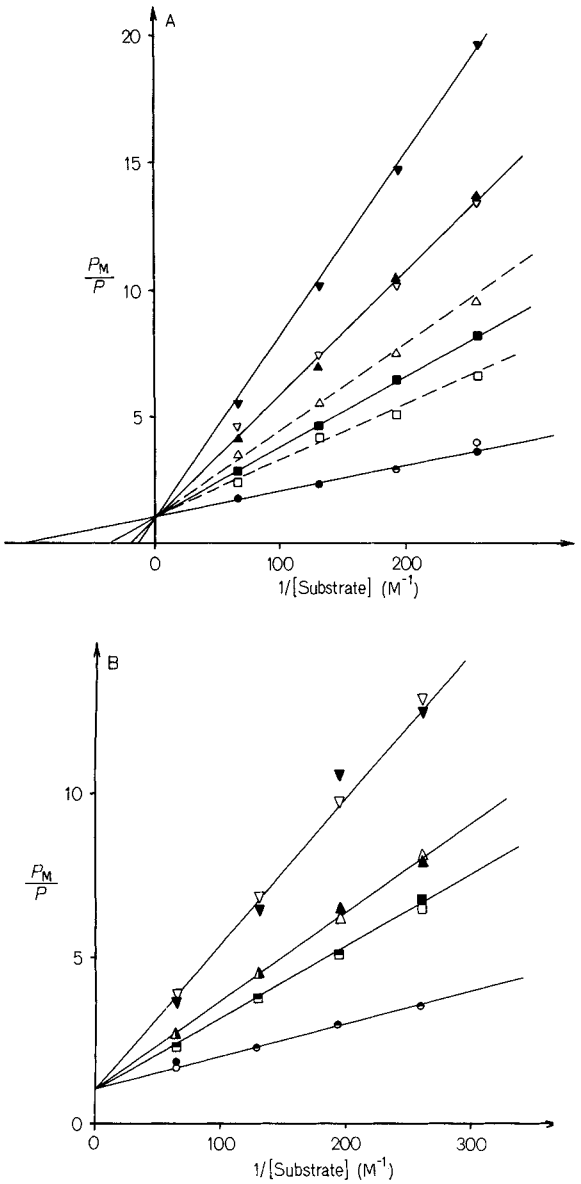

Fig. 1. *Theoretical and experimental plots of* P_M/P versus *1/[S] in the absence and in the presence of various concentrations of carbenicillin (A) and penicillin V (B).* Filled symbols = theoretical plots; open symbols = experimental plots [2]. (A) The carbenicillin concentrations were: zero (○, ●); 1.13 μM (□, ■); 2.25 μM (△, ▲) and 3.4 μM (▽, ▼). The experimental plot at [I] = 3.4 μM (▽) and the theoretical plot at [I] = 2.25 μM (△) were practically superimposable. (B) The penicillin V concentrations were: zero (○, ●); 0.53 μM (□, ■); 0.76 μM (△, ▲) and 1.52 μM (▽, ▼). In all cases the theoretical and experimental plots were superimposable. It should be noted that less than 13% of substrate was utilized in each case. Hence, it was assumed that the substrate concentration remained constant throughout the experiment

ed that the values of all the constants required for an accurate estimation of the theoretical P/P_M values from Eqn (7) were known. These theoretical values were then compared with those measured experimentally. As shown in Table 1, the agreement between the theoretical and experimental values was excellent with penicillin V (maximum variation: 8%), good with carbenicillin (maximum variation: 30%) and fair with benzylpenicillin (maximum variation: 50%). The differences between the theoretical and experimental values observed with benzylpenicillin were not surprising, since the k_3 and K values used for the theoretical estimation of P/P_M were obtained from experiments which for technical reasons had to be carried out at 25 °C, whereas the experimental estimation of P/P_M was carried out at 37 °C.

Not only with penicillin V, but also with carbenicillin and benzylpenicillin, both theoretical and experimental P/P_M values (Table 1) when plotted in a P_M/P *versus* 1/[S] graph, yielded straight lines converging on the ordinate axis. Fig. 1 shows plots obtained with carbenicillin and penicillin V. Moreover, the Dixon plots of both theoretical and experimental data yielded converging lines from which the following apparent K_i values were calculated: carbenicillin 0.73 μM (theoretical) and 1.05 μM (experimental); penicillin V 0.33 μM (theoretical and experimental); benzylpenicillin 61.5 nM (theoretical) and 80 nM (experimental). It should be noted that these apparent K_i values were significantly higher than those which would have been obtained from only the steady-state term of Eqn (7). Such apparent K_i values [$K_i = k_4K/(k_3 + k_4)$] were 0.17 μM, 93 nM and 10 nM for carbenicillin, penicillin V and benzylpenicillin respectively. As shown in Table 2, these differences were due to the fact that the two non-steady-state terms of Eqn (7) were not negligible with regard to the steady-state term. In the case of benzylpenicillin the contribution of the two non-steady-state terms were 3 to 4 times more important than that of the steady-state term.

Can the Non-Competitive Model be Excluded?

Carbenicillin was selected because at a concentration close to the K value it reacted with the R61 enzyme relatively slowly [1]. The rate constant k_a' for the formation of complex EI* was measured in the absence and in the presence of 6.5 mM and 13 mM substrate (Materials and Methods). The double-reciprocal plots of $1/k_a$ *versus* 1/[I] gave straight lines, which intersected the ordinate axis at values of 11 s when the substrate was absent from the reaction mixture, of 16.2 s in the presence of 6.5 mM substrate and of 14.7 s in the presence of 13 mM substrate.

It was impossible to determine whether these observed variations were significant or not. Because of the high Michaelis constant value (10 mM), substrate concentrations higher than those mentioned above should have been used. Since, however, both enzyme activity and penicillin binding are affected by increased ionic strengths [5, 6], the use of high substrate concentrations would also have given rise to

Table 2. *Values of the theoretical steady-state term (t_1) and of the theoretical non-steady-state terms ($t_2 + t_3$), according to Eqn (8)*

Antibiotic	Substrate	For $K/[I]$ =					
		96.4		48.4		32.1	
		t_1	$t_2 + t_3$	t_1	$t_2 + t_3$	t_1	$t_2 + t_3$
	mM						
Carbenicillin	3.9	0.048	0.075	0.026	0.047	0.018	0.033
	5.2	0.0627	0.094	0.035	0.062	0.024	0.044
	7.8	0.091	0.130	0.052	0.092	0.035	0.064
	15.5	0.165	0.200	0.096	0.154	0.068	0.117
		8.7×10^4		5.6×10^4		4.2×10^4	
		t_1	$t_2 + t_3$	t_1	$t_2 + t_3$	t_1	$t_2 + t_3$
Benzylpenicillin	2.15	0.013	0.048	0.010	0.035	0.007	0.0265
	4.3	0.026	0.090	0.018	0.067	0.011	0.052
	6.6	0.040	0.132	0.028	0.098	0.021	0.079
		For K/k_3 [I] =					
		1.29×10^3		0.89×10^3		0.45×10^3	
		t_1	$t_2 + t_3$	t_1	$t_2 + t_3$	t_1	$t_2 + t_3$
Penicillin V	3.9	0.094	0.058	0.072	0.052	0.042	0.035
	5.2	0.121	0.073	0.094	0.066	0.055	0.046
	7.8	0.170	0.095	0.135	0.088	0.080	0.065
	15.5	0.290	0.131	0.236	0.131	0.148	0.109

ambiguous results and the experiments were not attempted.

Furthermore, it should be noted that since all the data of Table 1 were obtained at $[I] \ll K$, the same P/P_M values would have been obtained on the basis of a non-competitive model if the additional condition $K' \approx K$ were also fulfilled (see mathematical treatments). It thus follows that a distinction between competitive and non-competitive models cannot be achieved on the basis of these kinetic experiments.

DISCUSSION

The main goal of this paper was to show that the mechanism proposed recently for the interaction between the R61 enzyme and β-lactam antibiotics [1] is compatible with Lineweaver-Burk plots exhibiting the characteristic features of a competitive inhibition. Indeed, both the experimental P/P_M values obtained previously [2] and the theoretical ones [based on Eqn (7) and on the constant values measured independently for the interaction between the enzyme and the antibiotic] coincide remarkably well. Competitive Lineweaver-Burk plots, however, are also obtained on the basis of a non-competitive model if the penicillin concentration were much smaller than the dissociation constant of the complex EI and the dissociation constant of the complex ESI. With such systems, competitive plots do not exclude the occurrence of a ternary complex ESI as classical competitive inhibitions do and, therefore, neither prove nor disprove that substrate and inhibitor compete for the same enzymic form.

The above conclusion strictly applies to the inhibition by β-lactam antibiotics of the exocellular DD-carboxypeptidase-transpeptidase from *Streptomyces* R61. Most likely, however, it also applies to other similar enzymes, such as the DD-carboxypeptidases from *Bacillus subtilis* and *Bacillus stearothermophilus*, for which both 'competitive' inhibition by β-lactam antibiotics and formation of rather stable enzyme · antibiotic complexes were described [7–10]. Contrary to a suggestion made by Blumberg and Strominger [7], the present studies support the idea that all DD-carboxypeptidases and transpeptidases are probably inhibited by penicillin according to the same general mechanism, although, depending upon the enzyme and/or the technique used, the inhibition may appear as 'competitive' (in Lineweaver-Burk plots) or as 'irreversible' (*i.e.* involving the formation of rather stable complexes EI*). Moreover, the same mechanism probably also applies to enzymes, such as the exocellular DD-carboxypeptidase-transpeptidase from *Streptomyces* R39, with which the formation of complex EI* is so fast and its decay is so slow that

the addition of increasing samples of penicillin to the enzyme results in the titration of one of the reactants by the other [11].

A structural analogy between penicillin and donor substrate cannot be justified on the basis of a 'competitive' inhibition. The type of inhibition of the exocellular R61 enzyme by penicillin, however, remains undetermined, since these studies do not allow us to distinguish between competitive and non-competitive models. Results obtained in other laboratories with the membrane-bound DD-carboxypeptidases from *Bacillus stearothermophilus* [12], *Streptococcus faecalis* [13] and *Proteus mirabilis* [14] would be best explained on the basis of a non-competitive model. These data, however, are still incomplete. Indeed, the stability of the complexes EI* and the molar ratios enzyme/antibiotic used in these experiments are not known. Under these conditions, a definite choice requires further work.

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