

## The Exocellular DD-Carboxypeptidase–Endopeptidase of *Streptomyces albus G*

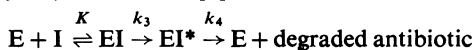
### INTERACTION WITH $\beta$ -LACTAM ANTIBIOTICS

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Kinetically, the three-step model proposed for the interaction between  $\beta$ -lactam antibiotics and the exocellular DD-carboxypeptidases–transpeptidases of *Streptomyces* R61 and *Actinomadura* R39 [Frère, Ghuysen & Iwatsubo (1975) *Eur. J. Biochem.* 57, 343–357; Fuad, Frère, Ghuysen, Duez & Iwatsubo (1976) *Biochem. J.* 155, 623–629] applies to the interaction between the much less penicillin-sensitive exocellular DD-carboxypeptidase–endopeptidase of *Streptomyces albus G* and at least phenoxymethylpenicillin, cephalothin and cephalosporin C. The penicillin resistance of the *albus G* enzyme is mainly due to the low efficiency with which the first reversible complex formed with the antibiotic (complex EI) undergoes transformation into a second more stable complex EI\*. Analysis of the ternary interaction between enzyme, *N*<sup>α</sup>*N*<sup>ε</sup>-diacetyl-L-lysyl-D-alanyl-D-alanine (Ac<sub>2</sub>-L-Lys-D-Ala-D-Ala) and cephalosporin C indicates a non-competitive mechanism.

The reaction between  $\beta$ -lactam antibiotics (I) and either the exocellular DD-carboxypeptidases–transpeptidases (E) excreted by *Streptomyces* R61 (the ‘R61 enzyme’) or by *Actinomadura* R39 (the ‘R39 enzyme’) is a three-step phenomenon:



where EI and EI\* are stoichiometric complexes, *K* is the dissociation constant of complex EI, and *k*<sub>3</sub> and *k*<sub>4</sub> are first-order rate constants (Frère *et al.*, 1975*b*; Fuad *et al.*, 1976). As a result of the interaction, penicillins are fragmented into *N*-acylglycine and *N*-formyl-D-penicillamine (Frère *et al.*, 1975*a*, 1976*a*; Adriaens *et al.*, 1978). Cephalosporins are also degraded, but the structure of the reaction products is unknown. The immobilization of both R39 and R61 enzymes in the form of complexes EI\* is a rapid reaction and the complexes thus formed exhibit rather high stability. Altogether these two properties are the cause of the high penicillin sensitivity of the two enzymes. In marked contrast, the exocellular DD-carboxypeptidase–endopeptidase excreted by *Streptomyces albus G* (the ‘*albus G* enzyme’; Duez *et al.*, 1978) requires high concentrations of penicillins to be inhibited (Leyh-Bouille *et al.*, 1970). On the basis of the mechanism proposed for the interaction between penicillin and the R61 and R39 enzymes, penicillin resistance may be caused by (1) a poor recognition of the antibiotic molecule by the enzyme (i.e. a high *K* value), (2) a slow formation of complex EI\* (i.e. a low *k*<sub>3</sub> value) and (3) a fast turnover of the antibiotic

molecule (i.e. a high *k*<sub>4</sub> value). Note that with unchanged *K* and *k*<sub>3</sub> values, and at a given antibiotic concentration, the higher the *k*<sub>4</sub> value the higher is the amount of enzyme that remains active at the steady state. This report describes experiments that were devised to establish the cause of the low penicillin sensitivity of the *albus G* enzyme.

### Materials and Methods

#### Antibiotics

Phenoxymethylpenicillin was a gift from Professor H. Vanderhaeghe, Katholieke Universiteit, Leuven, Belgium. Cephalosporin C and cephalothin were gifts from Lilly Laboratories, Indianapolis, IN, U.S.A.

#### Enzymes

The *albus G* enzyme used was that obtained after step 5 of the purification procedure described in the preceding paper (Duez *et al.*, 1978). In this preparation, the enzyme occurs in two forms: enzyme II (70% of the total amount) and enzyme I (30%). Except for the effect caused by sodium dodecyl sulphate in polyacrylamide-gel electrophoresis, enzymes I and II are identical in all other respects, including their (low) sensitivity to cephalosporin C (Duez *et al.*, 1978). The enzyme preparation is devoid of  $\beta$ -lactamase activity (less than  $1 \times 10^{-7}$  i.u./mg of protein). Penicillinase Riker (Neutropen; EC 3.5.2.6) was purchased from Serva Feinbiochemica (D-6900 Heidelberg, Germany).

### Assay of DD-carboxypeptidase activity

Enzyme and 1.7 mM- $Ac_2$ -L-Lys-D-Ala-D-Ala were incubated together at 37°C in 10 mM-Tris/HCl buffer, pH 8.0, containing 5 mM-MgCl<sub>2</sub>. The amount of C-terminal D-alanine liberated was measured enzymically (Frère *et al.*, 1976b).

### Interaction between enzyme and $\beta$ -lactam antibiotics

**Determination of the  $k_4$  values for the breakdown of complexes EI\*.** (a) Interaction with phenoxymethylpenicillin. Enzyme (25  $\mu$ g of protein/ml final concentration) and 16 mM-phenoxymethylpenicillin were incubated together for 4 h at 37°C in 50  $\mu$ l of 10 mM-Tris/HCl buffer (pH 8.0)/5 mM-MgCl<sub>2</sub>. Part of the enzyme (about 50% of the initial amount) was inactivated in the form of complex EI\*. The reaction mixture was supplemented with 1 i.u. of penicillinase (to destroy the excess of antibiotic) and maintained at 37°C (breakdown of complex EI\*). After increasing times, 5  $\mu$ l samples were removed and the extent of enzyme recovery was estimated by further incubation for 20 min at 37°C in the presence of 1.7 mM-substrate (for further details, see Frère *et al.*, 1975b).

(b) Interaction with cephalosporins. Enzyme (0.7 mg of protein/ml, final concentration) and 4 mM-cephalosporin were incubated together for 4 h at 37°C in 25  $\mu$ l of the same buffer as above. About 70 and 80% of the initial amount of enzyme was inactivated in the form of complex EI\* with cephalosporin C and cephalothin respectively. In some cases, the unbound cephalosporin was eliminated by filtration on a 10 ml column (0.6 cm  $\times$  1.0 cm) of Sephadex G-25 in 10 mM-Tris/HCl (pH 8.0)/5 mM-MgCl<sub>2</sub>. In other cases the reaction mixture was simply diluted with the buffer to 2.5 ml. This 100-fold dilution was sufficient virtually to prevent the free antibiotic from reacting both with the amount of enzyme not engaged in complex EI\* and with the free enzyme subsequently released through the breakdown of complex EI\*. Both techniques yielded the same results and, in both cases, the enzyme reactivation was followed as described above.

**Determination of the apparent rate constant  $k_a$  for the formation of complex EI\* in the presence of various concentrations of antibiotics.** Enzyme (25  $\mu$ g of protein/ml final concentration) and various antibiotic concentrations (from 9 mM- to 100 mM-phenoxymethylpenicillin; from 0.8 mM- to 16 mM-cephalothin; from 0.3 mM- to 16 mM-cephalosporin C) were incubated together at 37°C in 25  $\mu$ l of 10 mM-Tris/HCl (pH 8.0)/5 mM-MgCl<sub>2</sub>. On the basis of the inactivation of the enzyme, formation of complex EI\* was followed by measuring the residual enzyme activity on 1  $\mu$ l samples removed after increasing times of incubation (up to 60 min). For this purpose, the 1  $\mu$ l samples were diluted with 30  $\mu$ l of buffer and incubated

with 1.7 mM- $Ac_2$ -L-Lys-D-Ala-D-Ala for 20 min at 37°C. For the experiments carried out with phenoxymethylpenicillin, the incubation mixtures were supplemented with 1 i.u. of penicillinase. Penicillinase Riker has a very low activity on cephalosporins; for the experiments carried out with these last-named antibiotics, the 31-fold dilution of the samples was sufficient to prevent further reaction between free enzyme and free antibiotic. The antibiotic concentrations used were always much higher than the enzyme concentration, but the apparent rate-constant ( $k_a$ ) values were not much larger than the  $k_4$  values and the equation (Frère *et al.*, 1975b):

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

where  $[E_0]$  = total concentration of enzyme, should have been used to estimate the rate of formation of complex EI\*. However, the linearization of this equation would have required the measurement of the residual activity at the steady state, i.e. after very long incubation times of 7–8 h under which conditions a large proportion of the antibiotic would have been chemically degraded. Moreover, since the times used for the formation of the complexes EI\* (60 min, at the most) were always considerably shorter than the half-lives of these complexes (at least 130 min), the breakdown step was neglected and the  $k_a$  values were estimated on the basis of the simplified equation:

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

i.e. from plots of  $\ln(A_t/A_0)$  versus time, where  $A_t$  is the enzyme activity at time  $t$  and  $A_0$  the initial enzyme activity. It was computed that under these conditions and at the lowest antibiotic concentrations (I) used, the  $k_a$  value was at the most underestimated by 15%.

### Interaction between enzyme, $Ac_2$ -L-Lys-D-Ala-D-Ala and cephalosporin C

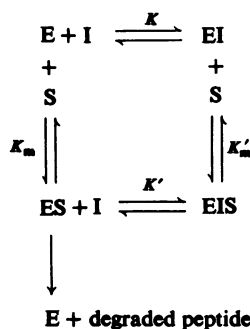
Enzyme (0.3  $\mu$ g of protein),  $Ac_2$ -L-Lys-D-Ala-D-Ala (from 0.48 to 1.66 mM) and cephalosporin C (from 0.23 to 1.10 mM) were incubated together for 15 min at 37°C in 110  $\mu$ l (final volume) of 10 mM-Tris/HCl (pH 8.0)/5 mM-MgCl<sub>2</sub> and the amount of D-alanine released was measured enzymically. In the present case, the usual procedure was slightly modified in that the final absorbance measurements were carried out on reaction mixtures supplemented with 200  $\mu$ l of methanol, instead of 300  $\mu$ l of methanol/water. Under the above conditions of incubation, the amount of complex EI transformed into complex EI\* was negligible (see the Results section); hence, assuming that formation of the ternary complex EIS occurred,

the reaction could be represented by a general non-competitive model (Scheme 1). The rate equation was:

$$\frac{1}{v} = \frac{1}{V} \left[ 1 + \frac{K_m}{[S]} \left( 1 + \frac{[I]}{K} \right) + \frac{[I]}{K'} \right]$$

The values of the various constants were estimated by computer analyses using the linear regression program devised previously (Schilf *et al.*, 1978). The weight  $w_i$  of each individual  $v_i$  value was estimated both as  $v_i$  and  $v_i^2$  and the minimized error function was:

$$\sum w_i^2 \left[ \frac{1}{v_i} - \left( \frac{1}{v_i} \right)_{th} \right]^2$$



Scheme 1. General non-competitive model for the interaction between enzyme (E), peptide substrate (S) and cephalosporin C (I) when a negligible amount of EI is transformed into EI\* (short incubation time)

$$K \times K'_m = K' \times K_m$$

where  $(1/v)_{th}$  is the theoretical value of  $1/v$  for given values of  $[I]$  and  $[S]$ . The Fisher-Snedecor variables  $F$  were used to estimate the probability with which the reaction proceeded either through a non-competitive model (Scheme 1) or through a competitive model (in which case, the ternary complex EIS was not formed).

## Results

### Breakdown of complexes EI\*: determination of $k_4$ values

Plots of  $\ln [1 - (A_t - A)/(A_0 - A)]$  versus time gave rise to straight lines ( $A_t$ , enzyme activity at time  $t$ ;  $A$ , residual enzyme activity after formation of complex EI\*, i.e., depending on the cases, after addition of penicillinase, after dilution of the reaction mixture or after Sephadex filtration;  $A_0$ , activity of an enzyme sample treated similarly, but in the absence of antibiotic). The  $k_4$  values thus obtained and the corresponding half-lives of the complexes EI\* are given in Table 1.

### Formation of complexes EI\*: determination of $K$ and $k_3$ values

Plots of  $\ln A_t/A_0$  versus time were straight lines and the plots of  $k_a$  versus  $[I]$  showed deviations from linearity at high  $[I]$  values. The  $K$ ,  $k_3$  and  $k_3/K$  ratio values were obtained from the reciprocal plots  $1/k_a$  versus  $1/[I]$  (Fig. 1 and Table 1). For comparison purposes, Table 1 also gives the  $K$ ,  $k_3$ ,  $k_3/K$  and  $k_4$  values that characterize the interactions between either the R39 enzyme or the R61 enzyme and some  $\beta$ -lactam antibiotics (Frère *et al.*, 1975b; Fuad *et al.*, 1976).

Table 1. Values of the constants involved in the interaction between  $\beta$ -lactam antibiotics and the exocellular *albus* G, R61 and R39 DD-carboxypeptidases

Data for enzymes R61 and R39 were selected from Frère *et al.* (1975b) and Fuad *et al.* (1976) respectively. Data for *albus* G enzyme were obtained as explained in the Results section.

Enzyme	Antibiotic	Formation of complex EI*			Breakdown of complex EI* (at 37°C)		
		$k_3/K$ ( $M^{-1} \cdot s^{-1}$ )	$K$ (mM)	$k_3$ ( $s^{-1}$ )	Temp. (°C)	$k_4$ ( $s^{-1}$ )	Half-life (min)
<i>albus</i> G	Phenoxymethylpenicillin	0.005	150	0.0008	37	$9 \times 10^{-5}$	130
	Cephalothin	0.06	9.5	0.0005	37	$3.3 \times 10^{-5}$	350
	Cephalosporin C	0.06	1.6	0.0001	37	$8 \times 10^{-5}$	145
R61	Cephaloglycine	22	0.4	0.009	37	$3 \times 10^{-6}$	3800
	Ampicillin	107	7.2	0.77	37	$1.4 \times 10^{-4}$	82
	Carbenicillin	820	0.11	0.09	37	$1.4 \times 10^{-4}$	82
	Cephalosporin C	1150	>1	>1	37	$1 \times 10^{-6}$	10000
	Phenoxymethylpenicillin	1500	>1	>1	37	$2.8 \times 10^{-4}$	40
	Benzylpenicillin	13700	13	180	25	$1.4 \times 10^{-4}$	82
R39	Cephalosporin C	66000	0.19	12.5	37	$0.3 \times 10^{-6}$	38000

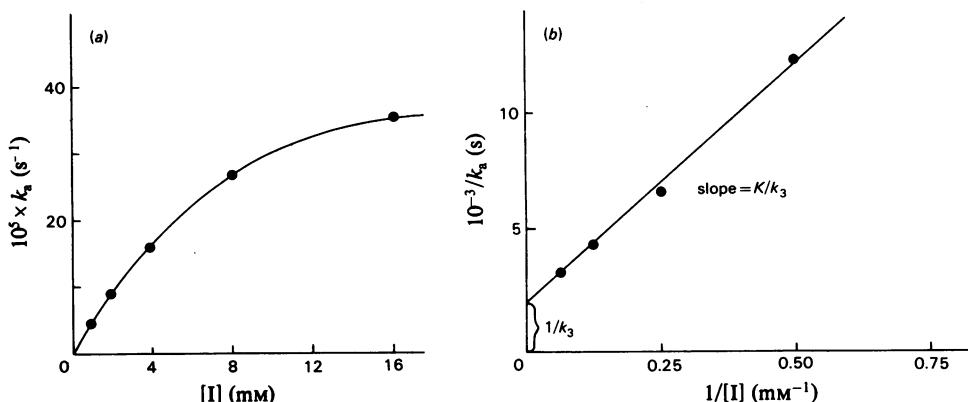


Fig. 1. Interaction between cephalothin and the albus G enzyme: effect of the antibiotic concentration  $[I]$  on the apparent rate constant  $k_a$  for the formation of complex  $EI^*$

(a) Direct plot of  $k_a$  versus  $[I]$ . (b) Reciprocal plot of  $1/k_a$  versus  $1/[I]$ .

Table 2. Values of the constants (in mM) involved in the non-competitive interaction between the albus G enzyme,  $Ac_2$ -L-Lys-D-Ala-D-Ala and cephalosporin C: computer analyses

On the basis of  $K \times K'_m = K' \times K_m$ , the  $K'_m$  values were 0.38 mM with  $w_1 = v_1$  and 0.39 mM with  $w_1 = v_1^2$ . The constants  $K$ ,  $K'$ ,  $K_m$  and  $K'_m$  refer to Scheme 1.

Assay no.	No. of experimental values	Mode of weighting	Fischer-Snedecor variable $F$ value	$K_m$	$K$	$K'$
1	19	$w_1 = v_1$	$23 > F_{99}$ (8.68)	0.56	1.14	0.83
2	20		$15 > F_{99}$ (8.53)	1.30	0.49	0.14
3	20		$F_{99}$ (8.53) $> 4.9 > F_{95}$ (4.49)	0.19	0.78	0.39
4	18		$11 > F_{99}$ (8.86)	0.70	0.40	0.26
5	20		$26 > F_{99}$ (8.53)	0.41	0.45	0.40
6	19		$9 > F_{99}$ (8.68)	0.28	0.31	0.40
Average $\pm$ S.E.				0.57	0.60	0.40
				$\pm 0.40$	$\pm 0.31$	$\pm 0.23$
1	19	$w_1 = v_1^2$	$18 > F_{99}$ (8.68)	0.63	1.48	0.70
2	20		$23 > F_{99}$ (8.53)	1.38	0.65	0.15
3	20		$F_{99}$ (8.53) $> 5.31 > F_{95}$ (4.49)	0.36	0.39	0.45
4	18		$11.5 > F_{99}$ (8.86)	0.55	0.60	0.40
5	20		$19 > F_{99}$ (8.53)	0.42	0.58	0.49
6	19		$17 > F_{99}$ (8.68)	0.26	0.30	0.30
Average $\pm$ S.E.				0.60	0.67	0.43
				$\pm 0.40$	$\pm 0.40$	$\pm 0.18$

#### Interaction between enzyme, $Ac_2$ -L-Lys-D-Ala-D-Ala and cephalosporin C

From the  $k_3$  value found for cephalosporin C (Table 1), it was computed that at substrate concentrations close to the  $K_m$  value (0.6 mM) and at cephalosporin C concentrations close to the  $K$  value, the amount of enzyme undergoing immobilization in the form of complex  $EI^*$  during the 15 min incubation at 37°C should be negligible, i.e. the inhibition of the enzyme should be exclusively due to the reversible formation of complexes  $EI$  and, possibly,  $EIS$ . Thus,

for example, at an  $Ac_2$ -L-Lys-D-Ala-D-Ala concentration equivalent to  $0.5K_m$  and a cephalosporin C concentration equivalent to  $5K$  and assuming a competitive interaction (no  $EIS$  formed), the amount of the reaction product  $P$  formed was estimated to be 97% of the theoretical amount that would be formed with a system where transformation of complex  $EI$  into complex  $EI^*$  would be completely excluded; assuming a non-competitive model, this value would still be higher. In agreement with these estimations, time-course experiments showed that during the

first 15 min of the incubation, the release of free D-alanine was linear; no sign of decrease of the reaction rate was observed, indicating that complex EI\* was not formed in detectable amounts. Six distinct experiments were carried out independently and the computer analyses of the data showed that the interaction was non-competitive with a level of confidence higher than 99% in five experiments and a level of confidence higher than 95% in one experiment (Table 2). The  $K$  value (i.e. the dissociation constant of the binary complex EI) thus obtained ( $0.60 \pm 0.31$  mM with  $w_1 = v_1$  and  $0.67 \pm 0.40$  mM with  $w_1 = v_1^2$ ) was very similar to that found by the direct procedure (1.6 mM; Table 1). The  $K$ ,  $K'$ ,  $K_m$  and  $K'_m$  constants that characterize the non-competitive interaction had similar values (about 0.5 mM).

### Discussion

Kinetically, the three-step model proposed for the interaction between  $\beta$ -lactam antibiotics and the penicillin-sensitive R61 and R39 enzymes (see the introduction) applies to the interaction between the much less penicillin-sensitive *albus* G enzyme and, at least, phenoxymethylpenicillin, cephalothin and cephalosporin C. Other  $\beta$ -lactams such as benzylpenicillin, cephalixin, carbenicillin, ampicillin, cloxacillin and methicillin were also examined, but their extremely low activity on the enzyme prevented any detailed study. On the basis of the data obtained with the three antibiotics studied, the *albus* G enzyme has a low penicillin sensitivity, not because the complexes EI\* are unstable (they have half-lives of 130 min or more), but because the formation of these complexes EI\* requires high antibiotic concentrations. With phenoxymethylpenicillin, this relative lack of reactivity can be attributed in part to a high  $K$  value, but, in all cases, it is clear that the main cause of penicillin resistance is very low  $k_3$  values. The importance of this latter parameter is also well emphasized when the  $k_3/K$  value for the interaction between the *albus* G enzyme and phenoxymethylpenicillin ( $0.005 \text{ M}^{-1} \cdot \text{s}^{-1}$ ) is compared with that for the interaction between the R61 enzyme and the same antibiotic ( $1500 \text{ M}^{-1} \cdot \text{s}^{-1}$ ) (Table 1): the  $K$  value for the *albus* G enzyme is at the most 150 times higher than that for the R61 enzyme, but the  $k_3$  value for the *albus* G enzyme is at least 1250 times smaller than that for the R61 enzyme. Similarly, the decreased sensitivity of the R61 enzyme to carbenicillin, ampicillin and cephaloglycine, when compared with benzylpenicillin, is also due to decreased  $k_3$  values (Table 1). The observations support Rando's (1975) idea that penicillin is a ' $k_{\text{cat}}$ ' inhibitor or a 'suicide' substrate; following this

view, the consequence of a decreased velocity of the 'suicide' step, i.e. a decreased  $k_3$  value, is, of course, a decreased inhibitory activity of the antibiotic molecule. Rapid breakdown of complex EI\* resulting in a rapid turnover of the antibiotic molecule leads also to penicillin resistance. This latter mechanism was the cause of the relatively low sensitivity to benzylpenicillin of the DD-carboxypeptidase of the unstable L-form of *Proteus mirabilis* (half-life of the complex: 3.5 min at 37°C; Martin *et al.*, 1976).

Because of the very low  $k_3$  value for the interaction between the *albus* G enzyme and cephalosporin C, the ternary interaction between enzyme, cephalosporin C and  $\text{Ac}_2\text{-L-Lys-D-Ala-D-Ala}$  could be studied by steady-state kinetics under conditions where the EI\* complex was virtually not formed. The interaction is non-competitive and the  $K$ ,  $K'$ ,  $K_m$  and  $K'_m$  constants exhibit similar values (about 0.5 mM). The DD-carboxypeptidase of the unstable L-form of *Proteus mirabilis* (Martin *et al.*, 1976; Schilf *et al.*, 1978) provides another example of a non-competitive interaction between DD-carboxypeptidase, penicillin and substrate. In this latter case, however, both formation and breakdown of complexes EI\* are fast processes.

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