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

INTERACTION WITH β-LACTAM ANTIBIOTICS

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Kinetically, the three-step model proposed for the interaction between β-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, $N^{\alpha}N^{\varepsilon}$-diacetyl-L-lysyl-D-alanyl-D-alanine (Ac_2-L-Lys-D-Ala-D-Ala) and cephalosporin C indicates a non-competitive mechanism.

The reaction between β-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:

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

where EI and EI* are stoicheiometric complexes, K is the dissociation constant of complex EI, and k_3 and k_4 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_3 value) and (3) a fast turnover of the antibiotic molecule (i.e. a high k_4 value). Note that with unchanged K and k_3 values, and at a given antibiotic concentration, the higher the k_4 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 β-lactamase activity (less than 1×10^{-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 1.7mM-Ac_2-L-Lys-D-Ala-D-Ala were incubated together at 37°C in 10mM-Tris/HCl buffer, pH8.0, containing 5mM-$MgCl_2$. The amount of *C*-terminal D-alanine liberated was measured enzymically (Frère *et al.*, 1976*b*).

Interaction between enzyme and β-lactam antibiotics

Determination of the k_4 values for the breakdown of complexes EI.* (*a*) Interaction with phenoxymethylpenicillin. Enzyme (25μg of protein/ml final concentration) and 16mM-phenoxymethylpenicillin were incubated together for 4h at 37°C in 50μl of 10mM-Tris/HCl buffer (pH8.0)/5mM-$MgCl_2$. Part of the enzyme (about 50% of the initial amount) was inactivated in the form of complex EI*. The reaction mixture was supplemented with 1i.u. of penicillinase (to destroy the excess of antibiotic) and maintained at 37°C (breakdown of complex EI*). After increasing times, 5μl samples were removed and the extent of enzyme recovery was estimated by further incubation for 20min at 37°C in the presence of 1.7mM-substrate (for further details, see Frère *et al.*, 1975*b*).

(*b*) Interaction with cephalosporins. Enzyme (0.7mg of protein/ml, final concentration) and 4mM-cephalosporin were incubated together for 4h at 37°C in 25μl 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 10ml column (0.6cm × 1.0cm) of Sephadex G-25 in 10mM-Tris/HCl (pH8.0)/5mM-$MgCl_2$. In other cases the reaction mixture was simply diluted with the buffer to 2.5ml. 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 (25μg of protein/ml final concentration) and various antibiotic concentrations (from 9mM- to 100mM-phenoxymethylpenicillin; from 0.8mM- to 16mM-cephalothin; from 0.3mM- to 16mM-cephalosporin C) were incubated together at 37°C in 25μl of 10mM-Tris/HCl (pH8.0)/5mM-$MgCl_2$. On the basis of the inactivation of the enzyme, formation of complex EI* was followed by measuring the residual enzyme activity on 1μl samples removed after increasing times of incubation (up to 60min). For this purpose, the 1μl samples were diluted with 30μl of buffer and incubated with 1.7mM-Ac_2-L-Lys-D-Ala-D-Ala for 20min at 37°C. For the experiments carried out with phenoxymethylpenicillin, the incubation mixtures were supplemented with 1i.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.*, 1975*b*):

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

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 7–8h 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* (60min, at the most) were always considerably shorter than the half-lives of these complexes (at least 130min), 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

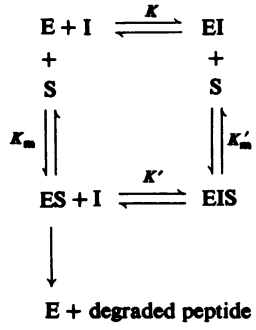
Enzyme (0.3μg of protein), Ac_2-L-Lys-D-Ala-D-Ala (from 0.48 to 1.66mM) and cephalosporin C (from 0.23 to 1.10mM) were incubated together for 15min at 37°C in 110μl (final volume) of 10mM-Tris/HCl (pH8.0)/5mM-$MgCl_2$ and the amount of D-alanine released was measured enzymically. In the present case, the usual procedure was slightly modified in that the final absorbance measurements were carried out on reaction mixtures supplemented with 200μl of methanol, instead of 300μl 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 $(1/v_i)_{th}$ 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 β-lactam antibiotics (Frère *et al.*, 1975*b*; Fuad *et al.*, 1976).

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

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

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

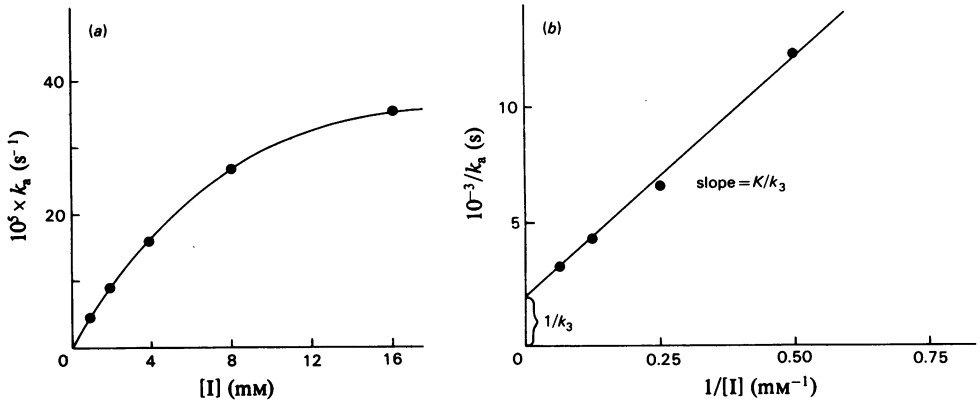


Fig. 1. *Interaction between cephalothin and the albus G enzyme: effect of the antibiotic concentration [I] on the apparent rate constant k_a for the formation of complex EI**
(*a*) Direct plot of k_a versus [I]. (*b*) Reciprocal plot of $1/k_a$ versus 1/[I].

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 $w_i = v_i$ and 0.39 mM with $w_i = v_i^2$. 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	$w_i = v_i$	$23 > F_{99}$ (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 ± S.E.				0.57 ±0.40	0.60 ±0.31	0.40 ±0.23
1	19	$w_i = v_i^2$	$18 > F_{99}$ (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 ± S.E.				0.60 ±0.40	0.67 ±0.40	0.43 ±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 15min 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 ± 0.31 mM with $w_i = v_i$ and 0.67 ± 0.40 mM with $w_i = v_i^2$) was very similar to that found by the direct procedure (1.6mM; Table 1). The K, K', K_m and K_m' constants that characterize the non-competitive interaction had similar values (about 0.5mM).

Discussion

Kinetically, the three-step model proposed for the interaction between β-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 β-lactams such as benzylpenicillin, cephalexin, 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 130min 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 ($0.005\,M^{-1}\cdot s^{-1}$) is compared with that for the interaction between the R61 enzyme and the same antibiotic ($1500\,M^{-1}\cdot s^{-1}$) (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 '$k_{cat.}$' 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: 3.5min 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 Ac_2-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 0.5mM). 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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References

Adriaens, P., Meesschaert, B., Frère, J. M., Vanderhaeghe, H., Degelaen, J., Ghuysen, J. M. & Eyssen, H. (1978) *J. Biol. Chem.* **253**, 3660–3665

Duez, C., Frère, J. M., Geurts, F., Ghuysen, J. M., Dierickx, L. & Delcambe, L. (1978) *Biochem. J.* **175**, 793–800

Frère, J. M., Ghuysen, J. M., Degelaen, J., Loffet, A. & Perkins, H. R. (1975*a*) *Nature (London)* **258**, 168–170

Frère, J. M., Ghuysen, J. M. & Iwatsubo, M. (1975*b*) *Eur. J. Biochem.* **57**, 343–351

Frère, J. M., Ghuysen, J. M., Vanderhaeghe, H., Adriaens, P., Degelaen, J. & De Graeve, J. (1976*a*) *Nature (London)* **260**, 451–454

Frère, J. M., Leyh-Bouille, M., Ghuysen, J. M., Nieto, M. & Perkins, H. R. (1976*b*) *Methods Enzymol.* **45**, 610–636

Fuad, N., Frère, J. M., Ghuysen, J. M., Duez, C. & Iwatsubo, M. (1976) *Biochem. J.* **155**, 623–629

Leyh-Bouille, M., Ghuysen, J. M., Nieto, M., Perkins, H. R., Schleifer, H. K. & Kandler, O. (1970) *Biochemistry* **9**, 2971–2975

Martin, H. H., Schilf, W. & Maskos, C. (1976) *Eur. J. Biochem.* **71**, 585–593

Rando, R. R. (1975) *Biochem. Pharmacol.* **24**, 1153–1160

Schilf, W., Frère, Ph., Frère, J. M., Martin, H. H., Ghuysen, J. M., Adriaens, P. & Meesschaert, B. (1978) *Eur. J. Biochem.* **85**, 325–330