

Interaction between Penicillin and the DD-Carboxypeptidase of the Unstable L-Form of *Proteus mirabilis* Strain 19

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Binding of penicillin to the DD-carboxypeptidase of the unstable spheroplast L-form of *Proteus mirabilis* results in the rapid formation of a modified enzyme-inhibitor complex which in turn undergoes rapid decay into reactivated enzyme and an antibiotically inactive penicillin degradation product. Major antibiotic metabolites recovered from such interactions were benzylpenicilloic acid and phenoxymethylpenicilloic acid from benzylpenicillin and phenoxymethylpenicillin, respectively, suggesting a second enzymic function of the DD-carboxypeptidase as a penicillinase of low efficiency. Statistical analyses made with the help of a linear regression program show that the enzyme interacts with the substrate UDP-*N*-acetylmuramoyl-L-alanyl-D- γ -glutamyl-(L)-*meso*-2,6-diaminopimelyl-(L)-D-alanyl-D-alanine and either benzylpenicillin or carbenicillin in a non-competitive manner.

In the presence of penicillin at high concentration (0.34 mM) the gram-negative bacterium *Proteus mirabilis* is able to grow as an unstable L-form and to synthesize a cell-wall peptidoglycan of apparently normal chemical composition. Therefore, DD-carboxypeptidases and transpeptidases, the specific enzymic targets of the inhibitory action of penicillin on bacterial peptidoglycan synthesis, must continue to function in this organism in spite of the presence of the antibiotic [1,2]. A membrane-bound DD-carboxypeptidase of the unstable *Proteus* L-form has been highly purified. Its preliminary characterization indicated that the enzyme was only moderately sensitive to penicillin, with a low stability of the enzyme-inhibitor complex and an apparent non-competitive mode of inhibition [3].

The validity of these notions has been examined by computer analyses of the kinetics of interaction of the purified *Proteus* L-form DD-carboxypeptidase with benzylpenicillin and carbenicillin on the basis

of the model of reaction developed for the exocellular DD-carboxypeptidases-transpeptidases of *Streptomyces* strain R61 [4,5] and *Actinomadura* strain R39 [6]. We also describe the reaction products into which penicillin is degraded during this interaction.

MATERIALS AND METHODS

Enzyme

Two preparations of the DD-carboxypeptidase of the unstable spheroplast L-form of *P. mirabilis*, strain 19, were used. Preparation A had been obtained by affinity chromatography on ampicillin bound to succinyl-aminododecyl-cellulose [3]. Preparation B had been subsequently submitted to preparative isoelectric focusing on Sephadex G-75 with ampholine, pH 5–8, at 1200 V and 15 W for 36 h (isoelectric point pH 5.9, W. Schilf and H. H. Martin, unpublished data). On gel electrophoresis in the presence of sodium dodecylsulfate, the enzyme gave rise to a single protein band with an apparent M_r value of 43000. Both enzyme preparations A and B, when used for the experiments described here, had specific activities of about 0.2 and 0.75 U/mg protein, respectively. One U catalyses the release of 1 μ mol D-alanine

Abbreviations. UDP-MurNAc-pentapeptide or UDP-MurNAc-LAla-DGlu(A₂pm-DAla-DAla), UDP-*N*-acetylmuramoyl-L-alanyl-D- γ -glutamyl-(L)-*meso*-2,6-diaminopimelyl-(L)-D-alanyl-D-alanine; Hepes, *N*-2-hydroxymethylpiperazine-*N'*-2-ethanesulfonic acid; Genapol X-100, isotridecanolpolyglycol ether (\bar{n} = 10).

Enzymes. DD-Carboxypeptidase (EC 3.4.12.6); penicillinase or β -lactamase (EC 3.5.2.6).

per min from UDP-MurNAc-LAla-DGlu(A₂pm-DAla-DAla) under conditions of enzyme saturation. Enzyme assays were carried out either by enzymic determination of D-alanine liberated from non-radioactive substrate [2] or by measurement of the amount of D-[¹⁴C]alanine released from UDP-MurNAc-LAla-DGlu(A₂pm-DAla-D[¹⁴C]Ala).

β-Lactam Antibiotics

Radioactive β-lactams used were [¹⁴C]benzylpenicillin (with the label on the carbonyl group of the phenylacetyl side chain, 54 Ci/mol) from the Radiochemical Centre, Amersham and phenoxymethyl-[³H]penicillin (with the label on the β-methyl group, 100 Ci/mol); this tritiated β-lactam was a gift from Prof. E. P. Abraham [7]. ¹⁴C-labelled benzylpenicilloic acid was prepared from [¹⁴C]benzylpenicillin by reaction with penicillinase (penicillinase Riker purchased from Serva Feinbiochemica, Heidelberg, F.R.G.). Chromogenic cephalosporin 87-312 [3-(2,4-dinitrostyryl)-(6*R*,7*R*)-7-(2-thienylacetamido)-ceph-3-em-4-carboxylic acid, E isomer] was a gift from Dr O'Callaghan (Glaxo Research Ltd, U.K.) [8].

Penicillin Metabolites

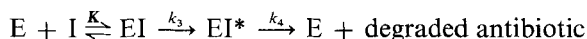
Separation of penicillin from its degradation products was performed by thin-layer chromatography on silica gel plates in three solvent systems: (a) 1-butanol/H₂O/acetic acid/ethanol (10/4/3/3; v/v/v/v); (b) H₂O/1-butanol/acetic acid (50/40/10, v/v/v, upper phase) and (c) chloroform/methanol/acetic acid (88/10/2, v/v/v) [9], and by high-voltage paper electrophoresis in collidine/acetic acid/H₂O (9.1/2.65/1000, v/v/v) at pH 6.5 [10].

Quantitative analysis of penicillin metabolites by column chromatography on Chromobeads type B cation-exchange resin was carried out as described by Adriaens et al. [11] and Coyette et al. [12].

COMPUTER ANALYSES OF INTERACTION BETWEEN ENZYME, SUBSTRATE AND PENICILLIN

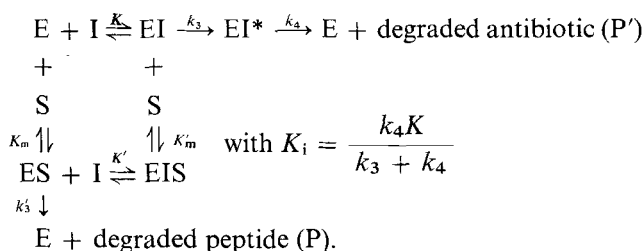
General Treatment: Non-competitive Model

The interaction between penicillin (I) and the extracellular DD-carboxypeptidases-transpeptidases (E) of both *Streptomyces* R61 and *Actinomadura* R39 is a three-step reaction:



with K = the dissociation constant of the binary complex EI and k_3 and k_4 = first-order rate constants [4,6].

Assuming that during the interaction between enzyme, penicillin and DAla-DAla-terminated peptide substrate (S), the ternary complex EIS is formed, a non-competitive model can be represented as follows:



The four steps in the loop are rapid equilibrium processes [5]. Under conditions where $[I] \gg [E]$ and for values of time such that the reaction has reached the steady state, the ratio V/v (with V the maximal velocity of formation of P at saturating concentration of substrate and in the absence of antibiotic, and v the velocity of the reaction either in the absence or the presence of penicillin and with nonsaturating concentrations of substrate) is given by the following equation [5]:

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

In order to estimate the V , K_m , K_i and K' values by computer analyses, Eqn (1) has been transformed into:

$$y_{th} = (1/v)_{th} = ax + bxz + c + dz \quad (2)$$

with y_{th} = the theoretical value of $1/v$, $x = 1/[S]$, $z = [I]$, $a = K_m/V$, $b = K_m/VK_i$, $c = 1/V$ and $d = 1/VK'$.

Estimation of the a, b, c, d Coefficient Values

The error function

$$\sum_{i=1}^{i=n} w_i^2 (y_i - y_{th})^2$$

with y_i = the experimental value of $1/v$, n = number of y_i values and w_i = the weight of each y_i value, has been minimized by calculating the a , b , c and d coefficients as shown below. In the present experiments, similar amounts of P were measured in all cases and for this purpose, the incubation times were adjusted depending upon the substrate concentrations. Consequently, all the experimental v values were affected by the same relative error and w_i was estimated as the reciprocal $1/y_i$.

On the basis that

$$\vec{p} = \begin{pmatrix} a \\ b \\ c \\ d \end{pmatrix}$$

Table 1. *Conditions of interaction between enzyme, substrate and penicillin*

In all cases, the incubation times at 37 °C were such that about the same amounts (2–4 nmol) of UDP-MurNAc-pentapeptide were hydrolyzed

Expt	Temp.	Enzyme prepn	Substrate concn	Antibiotic concn	Determination of free D-alanine	Number of measurements
	°C		mM	μM		
				Benzylpenicillin		
1	30	A	0.38, 0.57, 0.9, 1.32, 2.57	0, 0.5, 1, 2	radioactive	20
2	37	A	0.6, 0.9, 1.25, 2.5	0, 0.25, 0.5, 1, 1.5	enzymic	20
3	37	B	0.22, 0.33, 0.5, 0.8, 2, 5, 10	0, 0.3, 0.6, 0.9, 1.5	enzymic	35
				Carbenicillin		
4	37	B	0.33, 0.5, 0.8, 2, 5, 10	0, 5, 10, 20	enzymic	24

is the vector of the various coefficients to be determined,

$$\vec{y} = \begin{pmatrix} y_1 \\ \vdots \\ y_n \end{pmatrix}$$

is the vector of the measured variables, A the linkage matrix, A^T the transposed matrix A and w^2 the diagonal matrix of the w_i^2 values, the equation system representing the measurements is $\vec{y}_{th} = A\vec{p}$ and the solution to the problem of minimization is given by

$$\vec{p} = (A^T w^2 A)^{-1} A^T w^2 \vec{y}. \quad (3)$$

In order to solve the Eqn (3), a linear regression program was written in Fortran IV and the data were analyzed on a Modcomp II/10 minicomputer. The program is general and allows equations with k variables and l parameters to be analyzed. The same program also computes the residual variance (s_{OR}^2) and $\sqrt{s_{OR}^2 (A^T w^2 A)^{-1}_{ii}}$ values (see below). The inverted matrix was needed for this latter calculation and therefore a matrix inversion subroutine was used instead of a linear equation resolution subroutine.

Residual Variance and Confidence Interval

The residual variance is given by the equation

$$s_{OR}^2 = \frac{\sum w_i^2 y_i^2 - \sigma_1}{n - m}$$

with $\sigma_1 = \vec{p} A^T w^2 \vec{y}$ and m = number of parameters to be determined (i.e. four in the present case). The Student variable was then calculated for each parameter a , b , c and d on the basis of $(e - \varepsilon)/l_i$ with e = the experimental value of one given coefficient as determined by the program, ε = the real value of the same coefficient and $l_i = \sqrt{s_{OR}^2 [A^T w^2 A]_{ii}^{-1}}$. Finally, the confidence interval at 0.99 was computed

as $e - t_{99} \times l_i \leq \varepsilon_i \leq e + t_{99} \times l_i$ and the errors on the V , K_m , K_i and K' values were estimated from these interval values.

Comparison between Non-competitive and Competitive Models

Assuming that during the interaction between enzyme, penicillin and substrate, the ternary complex EIS is not formed, the model is competitive. The V , K_m and K_i values can also be measured on the basis of Eqn (2) in which, however, the dz term is eliminated ($K' = \infty$ and therefore $d = 0$).

In order to determine which of the non-competitive (NC) or competitive (C) models best fits the data, the Fisher-Snedecor variable (F) was calculated from

$$F = \frac{(t_{NC} - t_C)(n - 4)}{n - t_{NC}}$$

with $t_{NC} - t_C = (n - 3)(s_{OR}^2)_C - (n - 4)(s_{OR}^2)_{NC}$ and $n - t_{NC} = (n - 4)(s_{OR}^2)_{NC}$.

The higher the F value, the higher is the probability that a non-competitive model represents the real mechanism involved.

RESULTS

Interaction between Enzyme, Substrate UDP-MurNAc-LAla-DGlu(A_{2pm} -DAla-DAla) and β -Lactam Antibiotics

The amounts of D-alanine formed by the L-form DD-carboxypeptidase from the substrate was followed in 25 mM Na Hepes buffer pH 7.8 with 0.5% (w/v) Genapol X-100. Four sets of experiments were carried out under the conditions described in Table 1. In all cases where antibiotic was present, the plots of amount of D-alanine released versus time gave rise to straight lines which extrapolated at positive values

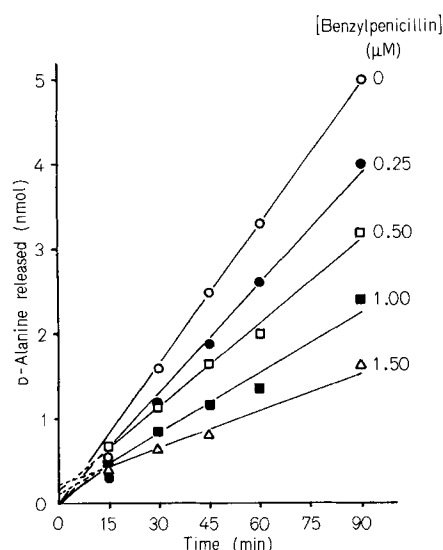


Fig. 1. Time course of release of D-alanine from 1.25 mM substrate UDP-MurNAc-pentapeptide by L-form DD-carboxypeptidase in 25 mM Na Hepes, pH 7.8, with 0.5% (w/v) Genapol X-100 at 37 °C and in the presence of various concentrations of benzylpenicillin and in the absence of antibiotic. The slopes of the linear part of the progress curves were computed by linear regression analysis

on the ordinate axis (Fig. 1). The burst thus observed at the onset of the reaction was due to the fact that it took about 2–3 min for the steady state to be established and it indicated that formation of complex EI* was a relatively slow process. After the burst, the steady-state velocity values (v) remained constant throughout the experiments. No sign of reacceleration of the reaction was observed indicating that, although the antibiotic was destroyed during the reaction, its concentration remained largely higher than the enzyme concentration.

Table 2 gives the residual variance values ($(s^2_{OR})_C$ and $(s^2_{OR})_{NC}$ for competitive and non-competitive models, respectively, and the Fisher-Snedecor variable F values. On the basis of the percentiles of the F distribution, the non-competitive model appears to be highly probable with levels of confidence of 90–95% for two sets of experiments and higher than 99% for two other sets of experiments.

Table 3 gives the values of the various parameters V , K_m , K_i and K' involved in a non-competitive type of interaction. From the corresponding confidence intervals ($t_{99} \times I_i$, also shown in Table 3), the K_m value for substrate is between 0.08 and 0.56 mM, the K_i value for benzylpenicillin is between 0.04 and 0.15 μ M and the K' value also for benzylpenicillin is lower than 2.7 μ M. With carbenicillin, the corresponding K_i value is 1.14 ± 0.50 μ M and the corresponding K' value is 36 ± 27 μ M.

The k_4 values for the breakdown of complexes EI* formed with benzylpenicillin and carbenicillin, respectively, were determined from the recovery times

Table 2. Comparison between competitive and non-competitive models for the interaction between enzyme, substrate and penicillin

The level of confidence for the choice of a non-competitive (NC) model was on the basis of the percentiles of the F distribution

Expt	$(s^2_{OR})_C$	$(s^2_{OR})_{NC}$	Fisher-Snedecor variable F	Level of confidence for the choice of NC model
				%
1	0.03191	0.01754	16	> 99
2	0.01342	0.01143	3.8	90–95
3	0.01343	0.01222	4.07	90–95
4	0.00996	0.00557	16.8	> 99

t in time courses of reappearance of DD-carboxypeptidase activity after enzyme inactivation by the corresponding β -lactamase and subsequent hydrolysis of the excess of antibiotic by penicillinase [3]. At 37 °C, the k_4 value was 3.3×10^{-3} s $^{-1}$ for benzylpenicillin (half-life of the complex = 3.5 min). From the K' and k_4 values and by assuming $k_3 \gg k_4$, the k_3/K value for formation of complex EI* was between 2 and 8×10^4 M $^{-1}$ s $^{-1}$.

Nature of the Degradation Product Arising from the Interaction between 14 C-Labelled and 3 H-Labelled Penicillins and the Enzyme

The degradation product was characterized subsequent to the breakdown of labelled penicillin and accumulation of the antibiotic metabolite in the course of the fast reaction of a given quantity of DD-carboxypeptidase with several consecutively added samples of penicillin. Alternatively, the antibiotic metabolite was obtained by release from the isolated enzyme-inhibitor complex EI*. At low temperature EI* was sufficiently stable to permit its separation from the reaction mixture.

In the first case, 118 mU DD-carboxypeptidase in 1 ml 50 mM Hepes buffer, pH 7.8, with 5% Genapol X-100, were incubated at 37 °C with 1.5 nmol [14 C]-benzylpenicillin (containing 81 nCi of radioactive label). Further samples of 0.8 nmol labelled penicillin were added at time intervals of 3.5 min with a total input of 43 nmol antibiotic containing 2.3 μ Ci of radioactivity within 3 h. The accumulated degradation product was purified and separated from reactivated DD-carboxypeptidase by gel filtration on a 15×1.5 -cm column of Sephadex G-25 fine in the cold and was characterized by thin-layer chromatography in solvents a, b and c and by high-voltage paper electrophoresis. One major radioactive metabolite was found. Its R_F values (solvent a, 0.58; b, 0.48 and c, 0.040) and migration distance of 33 cm after electrophoresis for 90 min at 60 V/cm coincided with those of authentic benzylpenicilloic acid. The above result

Table 3. Values with confidence intervals ($t_{99} \times 1_i$) of the various constants involved in the non-competitive interaction between enzyme, substrate and penicillin

Expt	Antibiotic	V	K_m	K_i	K'
		$\mu\text{mol min}^{-1} \text{mg}^{-1}$	mM	μM	
1	Benzylpenicillin	0.17 ± 0.065	0.62 ± 0.56	1.05 ± 1.35	1.26 ± 1.45
2	Benzylpenicillin	0.56 ± 0.24	1.95 ± 2.15	0.59 ± 0.55	1.02 ± 2.00
3	Benzylpenicillin	0.80 ± 0.11	0.32 ± 0.24	0.086 ± 0.06	4.7 ± 6.1
			$0.08 < K_m < 0.56$	$0.04 < K_i < 0.15$	$K' < 2.7$
4	Carbenicillin	0.78 ± 0.09	0.32 ± 0.15	1.14 ± 0.50	36 ± 27

was confirmed in a more detailed analysis of a sample of antibiotic metabolite corresponding to 55 nCi labelled antibiotic by column chromatography in a lithium citrate gradient on Chromobeads type B cation-exchange resin [11,12]. Here again, benzylpenicilloic acid was identified as the main degradation product, but other metabolites such as phenylacetyl-glycine and phenylacetic acid were also present in smaller amounts.

For the preparation of the antibiotic metabolite by release from the isolated EI* complex, 55 mU DD-carboxypeptidase in 1 ml 50 mM Hepes buffer, pH 7.8, containing 5% (w/v) Genapol X-100, were incubated for 10 min at 37 °C with either 30 nmol ^{14}C -labelled benzylpenicillin containing 1.63 μCi of radioactivity or 45 nmol phenoxymethyl ^3H penicillin with 4.5 μCi of radioactivity. Then the mixture were cooled to 4 °C and the EI* complexes formed were separated from unbound labelled antibiotic by gel filtration on a 15 \times 1.5-cm column of Sephadex G-25 fine in the cold. Release of the antibiotic metabolites from the complexes was obtained by incubation for 20 min at 37 °C.

Analysis of the penicillin degradation products was carried out by chromatography on a Chromobeads type B cation-exchange column as described. Quantitative yields of radioactive products varied in different experiments but [^{14}C]benzylpenicilloic acid (85% of the radioactivity extracted by ethyl acetate in one experiment) and phenoxymethyl ^3H penicilloic acid (80% of the radioactivity extracted), respectively, were the predominating metabolites in all cases. In addition, smaller amounts of other radioactive compounds were also obtained, such as phenylacetyl-glycine (8%) and phenylacetic acid (6%) from [^{14}C]benzylpenicillin and *N*-formyl-D-penicillamine (7%) from phenoxymethyl ^3H penicillin. However, minor compounds were also present in comparable amounts in a reaction mixture where [^{14}C]benzylpenicillin had been converted quantitatively into benzylpenicilloic acid by the action of penicillinase with or without further addition of DD-carboxypeptidase. Therefore, these compounds may be products of a further

metabolism of penicilloic acid formed during the various manipulations and/or may be present as contaminants of the labelled penicillins, as has been shown for phenylacetic acid.

From the foregoing, the action of *Proteus* L-form DD-carboxypeptidase on penicillins can be interpreted as that of a slow penicillinase. It is unlikely that the slow production of penicilloic acid by DD-carboxypeptidase is due to the presence of trace amounts of another classical β -lactamase in the purified DD-carboxypeptidase preparation, because β -lactamase has previously been found to be totally absent from spheroplasts of the unstable *Proteus* L-form and from crude membrane-bound or solubilized DD-carboxypeptidase of this organism. Also, even on prolonged interaction purified L-form DD-carboxypeptidase did not give any detectable reaction with chromogenic cephalosporin 87-312, [3-(2,4-dinitrostyryl)-(6*R*, 7*R*)-7-(2-thienylacetamido)-ceph-3-em-4-carboxylic acid, E isomer], a highly sensitive detector of β -lactamases [8].

DISCUSSION

In previous work with the purified L-form DD-carboxypeptidase, Lineweaver-Burk plots with the characteristics of non-competitive inhibition by penicillin and carbenicillin had already been obtained using individual measurements of initial velocities [3]. The present statistical analyses made with the help of a linear regression program especially devised for the purpose (and referred to as BRUNO program) confirmed these preliminary data and hence support the conclusion that the ternary complex EIS is actually formed during the interaction between enzyme, substrate and β -lactam antibiotic. The present study did not establish whether or not this ternary complex EIS could give rise to degraded peptide, P. Dixon plots (not shown) of the actual experimental data did not show signs of hyperbolic non-competitive inhibition, suggesting that EIS was a dead-end complex. With all the DD-carboxypeptidases-transpeptidases formerly examined, including both exocellular R61

and R39 enzymes, the inhibition appeared to be of the competitive type at least by graphical analyses [4–6]. As amply discussed elsewhere [13,14], these results are not persuasive. They only indicate that under the experimental conditions used (or rather imposed because of technical limitations), $[EIS]$ is $\ll [EI] + [EI^*]$.

The interaction between the L-form DD-carboxypeptidase and penicillin is characterized by a rapid formation of complex EI^* (high k_3/K values) and the subsequent dissociation of this labile complex at fairly high velocity with enzyme reactivation and conversion of penicillin to penicilloic acid. This rapid degradation of penicillin confers to the DD-carboxypeptidase a high resistance to penicillin which may be relevant to the continued function in the L-form organism during growth in the presence of penicillin. Penicillin binding protein 5 of *Escherichia coli* K12 (a DD-carboxypeptidase) also forms with benzylpenicillin a transient complex of short half-life (5 min at 30 °C) [15].

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