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Fragmentation of benzylpenicillin after interaction with the exocellular DD-carboxypeptidase-transpeptidases of *Streptomyces* R61 and R39

THE killing target of penicillin in bacteria is a membrane-bound transpeptidase which catalyses peptide cross linking during wall peptidoglycan synthesis[1,2]. *Streptomyces* R61 and R39 excrete during growth DD-carboxypeptidase-transpeptidase enzymes[3,4] which seem to be soluble forms of the corresponding membrane-bound transpeptidases[5]. The exocellular enzymes (E) convert penicillin (I) in to a chemically altered and biologically inactive compound (X)[6,7]. Kinetically, the simplest mechanism[5] for the conversion of I into X is

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

The first step, a rapid equilibrium process, leads to the formation of an equimolar and inactive enzyme–antibiotic complex EI. This complex isomerises into a modified complex EI* which, in turn, undergoes irreversible breakdown. If the experiment is carried out in conditions in which the enzyme is stable, the enzyme is reactivated and recovers its initial penicillin sensitivity. The breakdown of complex EI* is a slow process. At 37 °C and in 10 mM Na phosphate buffer, *p*H 7.0 (in which conditions the R61 enzyme is stable) the half life of the R61 enzyme–benzylpenicillin EI* complex is 80 min (ref. 7). At 37 °C and in 0.1 M Tris-HCl buffer, *p*H 7.7, containing 0.1 M NaCl and 0.05 M $MgCl_2$ (in which conditions the R39 enzyme is stable), the half life of the R39 enzyme–benzylpenicillin is 4,250 min (ref. 6). As the enzyme is reactivated during the process, no enzyme is irretrievably lost on reaction with penicillin and after several cycles of inactivation and reactivation, both the enzyme and the accumulated X compound can be reisolated. This technique was applied to ^{14}C-benzylpenicillin (benzyl labelled) and the ^{14}C-X compound produced was isolated.

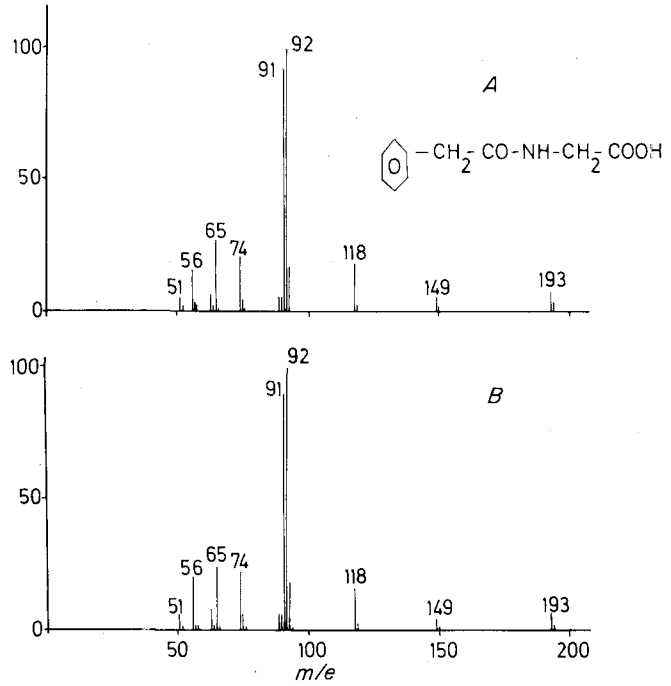

Fig. 1 Mass spectra of authentic phenylacetylglycine (*A*, molecular weight = 193) and X compound (*B*). ^{14}C-X (0.5 μeq) was dissolved in 100 μl of 6 N HCl and extracted, at room temperature, with 300 μl of ethyl acetate (yield of the extraction 60% in terms of d.p.m.). Mass spectrography was carried out on a sample of the extract containing 5 neq of ^{14}C-X and compared with that given by 1 μg of phenylacetylglycine. Spectra were recorded at 70 eV with an ionisation current of 60 μA. The sample was directly introduced (193 : molecular ion M^+).

A solution of R61 enzyme (0.9 μmol; molecular weight 38,000) in 20 ml of phosphate buffer was supplemented at 37 °C with 0.72 μmol of ^{14}C-benzylpenicillin (0.7 mCi $mmol^{-1}$). Every 30 min, an additional 0.144 μmol of ^{14}C-benzylpenicillin was added until a total amount of 29 μmol of antibiotic was used. Filtration on Sephadex G-25 and elimination of phosphate by addition of acetone at 0 °C yielded 19 μeq of purified ^{14}C-X. Similarly, a solution of R39 enzyme (11.7 nmol; molecular weight 53,000) in 100 μl of Tris-NaCl-$MgCl_2$ buffer was supplemented with 14.5 nmol of ^{14}C-benzylpenicillin (25 mCi $mmol^{-1}$) and the mixture was incubated for 51 h at 37 °C. Filtration on Sephadex G-25 yielded 4 neq of ^{14}C-X.

The ^{14}C-X obtained with the R61 enzyme was identified as ^{14}C-phenylacetylglycine on the following grounds. (1) Acid hydrolysis with 6 N HCl at 120 °C yielded glycine (yield 100% with the amino acid analyser and 76% after transformation into dinitrophenylglycine) and ^{14}C-phenylacetic acid (as revealed by thin-layer chromatography on Silica-Gel plates using two solvents; Table 1). (2) ^{14}C-X and authentic phenylacetylglycine exhibited the same R_f values by thin-layer chromatography on Silica-Gel plates using three solvents (Table 1). (3) A mixture containing a few neq of ^{14}C-X and a 1,000-fold excess, on a molar basis, of non-radioactive phenylacetylglycine was submitted to three successive crystallisations from ethylacetate. Crystals thus obtained exhibited constant specific radioactivities. (4) The mass spectrum of ^{14}C-X was identical to that given by phenylacetylglycine (Fig. 1). (5) Both ^{14}C-X and phenylacetylglycine, esterified with CH_2N_2, had the same retention time (6 min) by gas–liquid chromatography (at 200 °C, with H_2 and N_2 flow rates of 2 and 30 ml min^{-1}, respectively, and by using a capillary SP1000 column of length 20 m). Similarly, the ^{14}C-X compound obtained with the R39 enzyme was also characterised as ^{14}C-phenylacetylglycine by cocrystallisation and cochromatography with authentic phenylacetylglycine.

We conclude, therefore, that by interacting with benzylpenicillin, both R61 and R39 exocellular enzymes split the antibiotic molecule and that one of the fragments is phenylacetylglycine. Evidently the methylene carbon atom of the glycine residue must arise from C_6 of the penicillin nucleus and, presumably, the C_7 is retained as the glycine carboxyl group (Fig. 2). The fate of the thiazolidine nucleus of the penicillin molecule has not yet been determined. Chemically, benzylpenicillin methyl ester can be degraded to methyl D-5,5-dimethyl-Δ^2-thiazoline-4-carboxylate in trifluoroacetic acid. The N-phenylacetylglycyl fragment was isolated by conversion to its N-benzylamide[8]. A possible mechanism for the action of the *Streptomyces* enzymes would be that the initial event is a rupture of the

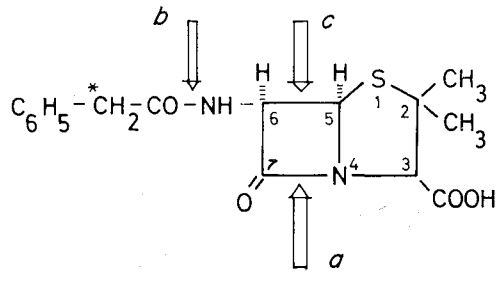

Fig. 2 ^{14}C-benzylpenicillin. *a*, Site of action of penicillinase (reaction product benzylpenicilloic acid); *b*, site of action of amidase (reaction product 6-aminopenicillanic acid); *c* and *a*, possible sites of action of the DD-carboxypeptidase-transpeptidases of *Streptomyces* R61 and R39 (reaction product phenylacetylglycine + unknown). *, [^{14}C].

Table 1 R_f values of ^{14}C-X and phenylacetylglycine and of HCl-hydrolysed ^{14}C-X and phenylacetic acid, by cochromatography on Silica-Gel G thin-layer plates

Enzyme used for the preparation of ^{14}C-X	Solvent	R_f values of ^{14}C-X	Added phenylacetylglycine	HCl-hydrolysed ^{14}C-X	Added phenylacetic acid
R61	*a*	0.77	0.76	0.80	0.80
	b	0.71	0.70		
	c	0.40	0.40	0.56	0.55
R39	*a*	0.78	0.77		
	b	0.71	0.69		
	c	0.36	0.36		

Solvents: *a*, 1-butanol–H_2O–acetic acid–ethanol: 10:4:3:3 (v:v:v:v); *b*, water–1-butanol–acetic acid: 50:10:10 (v:v:v) upper phase; *c*, chloroform–methanol–acetic acid: 88:10:2 (v:v:v).

Note that the R_f values of benzylpenicilloic acid are 0.65 in solvent *a* and 0.55 in solvent *b*. Benzylpenicilloic acid is immobile in solvent *c*.

β-lactam amide bond (Fig. 2; arrow *a*) with attachment of C_7 to some active group in the enzyme. By a process of β elimination, in which functional groups of the enzyme could participate, the link between C_5 and C_6 would become a double bond and further degradation would result in removal of the thiazolidine moiety (Fig. 2; arrow *c*). Release of the phenylacetylglycine would then occur. There may be other possible mechanisms and the problem is being studied at present.

Whatever the mechanism, however, our studies point to the great importance of the nature of the substituents on C_6 of the penicillin molecule. Substitution at C_6 drastically reduces the activity of the β-lactam antibiotics in all cases tested; 6-methoxypenicillin derivatives, however, remain better inhibitors than the corresponding 6-methyl derivatives[9]. These studies may also be related with the observations of Schmid and Plapp[10] that binding of penicillin to *Proteus mirabilis* was inhibited by phenylacetylglycine and that in the presence of this compound, formation of sphaeroplasts was prevented. Experiments have shown that the extracted and partially purified membrane-bound transpeptidases from *Streptomyces* R61 and *S. rimosus*[5] also perform fragmentation of benzylpenicillin with formation of phenylacetylglycine (M. Leyh-Bouille, J. Dusart and J. M. Ghuysen, unpublished). Moreover, penicillin is known to be degraded into a biologically inactive compound by interacting with the membrane-bound DD-carboxypeptidases of various *Bacillus* spp.[11]. The fact that the membrane-bound targets of penicillin act as antibiotic-degrading compounds is important even if the overall process is slow. It leads to a better understanding of the phenomena of resistance which cannot be explained on the basis of production of either penicillinase or amidase (Fig. 2; arrows *a* and *b*, respectively). It may also be relevant to the necessity for the continuous supply of antibiotic during penicillin therapy.

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