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The penicillin-binding site in the exocellular DD-carboxypeptidase–transpeptidase of *Actinomadura* R39

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Heat denaturation and Pronase degradation of the complex previously formed between benzylpenicillin and the exocellular DD-carboxypeptidase–transpeptidase of *Actinomadura* R39 yields a heptapeptide H-Leu-Pro-Ala-Ser-Asn-Gly-Val-OH, where the benzylpenicilloyl group is ester-linked to the serine residue. This linkage is very labile and its hydrolysis causes the release of benzylpenicilloate. In contrast, the native benzylpenicilloyl–enzyme complex is very stable (half-life 70h at 37°C) and its breakdown proceeds via fragmentation of the bound benzylpenicilloyl group [Fuad, Frère, Ghuysen, Duez & Iwatsubo (1976) *Biochem. J.* **155**, 623–629].

The complexes formed by interaction between penicillin and the DD-carboxypeptidases have received much attention. With the exocellular DD-carboxypeptidase–transpeptidase of *Streptomyces* R61 (Frère *et al.*, 1976*a*; Degelaen *et al.*, 1979) and the membrane-bound DD-carboxypeptidases of *Bacillus subtilis* and *B. stearothermophilus* (Georgopapadakou *et al.*, 1977; Yocum *et al.*, 1979), it is known that: (1) penicillin acylates the enzymes and forms rather stable penicilloyl–enzyme complexes, (2) the penicilloyl group is ester-linked to an enzyme serine residue (as least as found after denaturation of the complexes) and (3) breakdown of the native complexes leads to enzyme regeneration and proceeds via fragmentation of the bound penicilloyl moiety. As shown with the *Streptomyces* R61 enzyme (Frère *et al.*, 1976*b*), this process implies splitting of the C-5–C-6 bond and protonation of C-6, causing the release of *N*-acylglycine and *N*-formyl-D-penicillamine.

The exocellular DD-carboxypeptidase–transpeptidase of *Actinomadura* R39 (in short, the R39 enzyme) resembles the R61 and *Bacillus* enzymes at least in two respects. First, the interaction between the R39 enzyme and β-lactam antibiotics leads to the formation of stable complexes in which the bound metabolite probably occurs in the form of a penicilloyl or cephalosporoyl derivative. Thus, in such complexes, cephaloglycine, cephalexin and cephalosporin C have their ε_{260} decreased to the same extent as that obtained after the action of β-lactamase (Fuad *et al.*, 1976), and nitrocefin has a $\varepsilon_{482}/\varepsilon_{386}$ ratio of 2.40, which is that obtained after the action of β-lactamase (Frère *et al.*, 1974). Secondly, spontaneous breakdown of the native penicilloyl-R39 enzyme complex also proceeds (slowly) via fragmentation of the bound metabolite (Frère *et al.*, 1975). The present paper describes experiments which were carried out to characterize the amino acid residue which may serve as the penicillin attachment site in the R39 enzyme.

Materials and methods

The R39 enzyme, the [^{14}C]benzylpenicillin (57mCi/mmol) and the [^{14}C]benzylpenicilloyl–R39 enzyme complex were those previously used (Fuad *et al.*, 1976). Trypsin treated with 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one (TPCK-trypsin) was purchased from Worthington. Pronase was from Sigma. The following chromatography solvents were used: I, butan-1-ol/acetic acid/pyridine/water (15:3:10:12, by vol.); II, chloroform/methanol/acetic acid (44:5:1, by vol.); III, butan-1-ol/water/acetic acid/ethanol (10:4:3:3, by vol.). T.l.c. was performed on silica gel G-60 plates. High voltage electrophoreses were carried out with a Gilson electrophorator model DW at 60V·cm^{-1}, in collidine/acetic acid/water (33:13:5000, by vol.) (pH6.5), using Whatman 3MM papers and refrigerated tanks. Peptides were stained with fluorescamine (F. Hoffmann-La Roche Inc., Basel, Switzerland) as described by Vandekerckhove & Van Montagu (1974). A Packard Tri-Carb 2425 liquid-scintillation spectrometer and a Packard radiochromatogram scanner model 7201 were used

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for the radioactivity measurements. Amino acid analyses (after hydrolysis of the samples in azeotropic HCl for 24h at 105°C) were performed with a Beckman Multichrom 4255 automatic amino acid analyser. Amino acid sequencing was carried out according to the micromethod of Bruton & Hartley (1970). The dansyl amino acids were characterized as described by Weiner *et al.* (1972) on 5cm × 5cm polyamide plates. Benzylpenicilloate was characterized by paper electrophoresis at pH 6.5 (mobility 25cm·h^{-1} at 60V·cm^{-1}) and by t.l.c. in both solvent II (R_F 0.03) and solvent III (R_F 0.56).

Results

Effects of heat denaturation and trypsin degradation on the stability of the [^{14}C]benzylpenicilloyl–R39 enzyme complex

Heat denaturation of the [^{14}C]benzylpenicilloyl–R39 enzyme complex (by boiling a solution of the complex for 1min in 10mM-sodium phosphate, pH 7.0) had two effects. (1) As determined by the rate of release of radioactivity, the denatured complex had a decreased half-life of 10h (instead of 70h for the native complex). (2) Spontaneous breakdown of the heat-denatured complex resulted in the release of [^{14}C]benzylpenicilloate (and did not proceed through fragmentation of the benzylpenicilloyl moiety as observed with the native complex). Note that denaturation by sodium dodecyl sulphate (instead of heat) also caused a decreased stability of the complex (half-life 150min at 37°C; Frère *et al.*, 1974).

Degradation of the heat-denatured [^{14}C]benzylpenicilloyl–R39 enzyme with TPCK-trypsin further destabilized the bond between the peptide and the benzylpenicilloyl group. The heat-denatured complex (2nmol in 30µl of 10mM-Tris/HCl, pH 8.0) was supplemented with 1.5µg of trypsin. After 2h at 37°C, a fresh sample of 1.5µg of trypsin was added and the mixture was further incubated for 2h. Analysis of the degradation products by paper electrophoresis at pH 6.5 showed that all the radioactivity was present in the form of [^{14}C]-benzylpenicilloate. However, if a higher amount of trypsin (15µg) was used, so that the incubation at 37°C could be decreased to 30min (instead of 4h), electrophoresis of the degradation products permitted detection, in addition to [^{14}C]benzylpenicilloate, of a radioactively labelled, negatively charged peptide that had a mobility of 7cm·h^{-1} at 60V·cm^{-1}.

Isolation of a [^{14}C]benzylpenicilloyl–heptapeptide

Essentially, the same procedure as above was used except that Pronase was used instead of trypsin. Moreover, the proteolytic degradation at 37°C was made as short as possible and all the other operations were carried out at 4°C. The heat-treated complex (57nmol in 50µl of 10mM-sodium phosphate, pH 7.0) was incubated with 240µg of Pronase (two successive additions of 120µg) for 30min at 37°C. Filtration of the sample in water through a column (60cm × 1.0cm) of Biogel P-2 at 4°C yielded two radioactive fractions. The fraction with K_D 1.0 contained [^{14}C]benzylpenicilloate. The fraction with K_D 0.62 was lyophilized and submitted to paper electrophoresis for 2h at pH 6.5 and 60V·cm^{-1}. Half of the radioactivity initially present in the fraction with K_D 0.62 was recovered as [^{14}C]benzylpenicilloate and the other half was found to be associated with a negatively charged peptide (mobility 7.5cm·h^{-1} at 60V·cm^{-1}). In turn, this radioactive peptide was submitted to descending paper chromatography in solvent I at 4°C. Again, half of the radioactivity was recovered as [^{14}C]-benzylpenicilloate (which migrated close to the solvent front) and the other half as found to be associated with a peptide of R_F 0.64. This radioactive peptide was eluted with water at 4°C. In terms of radioactivity, the final yield was 7% of that present in the original complex. Chromatography of the radioactive peptide on Biogel P-2 (in water at 4°C) indicated a mol.wt. of approx. 1100. Its amino acid composition (Asx, 1.2; Ser, 0.6; Pro, 1.0; Gly, 1.0; Ala, 0.8; Val, 1.1; Leu, 0.6) suggested that it was a heptapeptide consisting of one residue each of Asx, Ser, Pro, Gly, Ala, Val and Leu. On the basis of this composition, the corresponding penicilloyl–heptapeptide should have a theoretical mol.wt. of 970.

Evidence for the presence of an ester linkage in the [^{14}C]benzylpenicilloyl–heptapeptide fragment

The heat-denatured [^{14}]benzylpenicilloyl–R39 enzyme complex (45nmol) was treated with Pronase, the degradation products were filtered on Biogel P-2 (at 4°C) and the radioactive fraction with K_D 0.62 was lyophilized, as described in the preceding section. The fraction with K_D 0.62 was analysed by two-dimensional paper electrophoresis at pH 6.5 and 60V·cm^{-1} under the following conditions. A first electrophoresis was carried out on a Whatman 3MM paper strip (5cm × 150cm) for 30min. The paper strip (from which that part containing free [^{14}C]-benzylpenicilloate was eliminated) was exposed to NH_3 vapour for 16h at room temperature, dried at 80°C and sewn to a Whatman 3MM paper sheet (27cm × 150cm). A second electrophoresis was then carried out for 60min in a direction perpendicular to that used for the first one. Staining of the electrophoretogram with fluorescamine showed that all the peptides were positioned along one oblique line except one of them (Fig. 1). Obviously, this peptide (spot 1) had migrated as an acidic compound with a

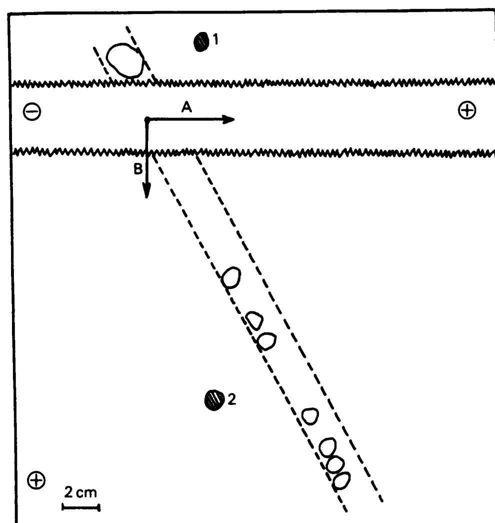

Fig. 1. *Purification of the penicillin-binding peptide by two-dimensional electrophoresis*
The first electrophoresis took place in direction A, the second in direction B. For details, see the text. Spot 1 was fluorescent and non-radioactive (the Heptapeptide). Spot 2 was radioactive and non-fluorescent (α-amide of [^{14}C]benzylpenicilloic acid).

mobility of 7.5 cm·h^{-1} during the first electrophoresis, but had been transformed during treatment with NH_3 so that it behaved as a neutral peptide during the second electrophoresis (its slight migration towards the cathode was due to electro-osmosis). In turn, scanning of the electrophoretogram permitted detection of one single radioactive compound (spot 2). This compound which, during the first electrophoresis, had migrated towards the anode with the same mobility (7.5 cm·h^{-1}) as that observed for peptide 1 before NH_3 treatment, migrated, during the second electrophoresis, with a mobility of 15 cm·h^{-1}, i.e. most likely that of the α-amide of benzylpenicilloic acid. Peptide 1, as it occurred in the fraction with K_D 0.62, thus behaved as a negatively charged benzylpenicilloyl–peptide. After NH_3 treatment, the same peptide 1 then behaved as a neutral, benzylpenicilloyl-free compound. The benzylpenicilloyl-free peptide 1 was eluted from the electrophoretogram; 7 nmol were obtained (yield 16%). Amino acid composition and sequencing studies gave the following primary structure: H-Leu-Pro-Ala-Ser-Asn-Gly-Val-OH. Residue 5 was assumed to be amidated, on the basis of the neutral character of the peptide at pH 6.5. Attachment of a penicillin molecule to this neutral heptapeptide, so that the derivative thus formed acquires one negative charge, exhibits low stability and breaks down in water with release of benzylpenicilloate, necessarily involves penicilloylation of the serine residue via formation of an ester linkage.

Discussion

Heat treatment and proteolytic degradation (with either trypsin or Pronase) of the benzylpenicilloyl–R39 enzyme complex considerably labilizes the linkage between the bound metabolite and its attachment site on the enzyme. In spite of this high lability, a benzylpenicilloyl-heptapeptide complex (generated by Pronase treatment) has been isolated and the penicilloyl group has been shown to be ester-linked to its serine residue. Like the R61 and the *Bacillus* DD-carboxypeptidases, the R39 enzyme thus appears to be a serine DD-carboxypeptidase.

Spontaneous hydrolysis of the ester bond which links the benzylpenicilloyl group to the heptapeptide is rapid and leads to the release of benzylpenicilloate. In contrast, the native benzylpenicilloyl–enzyme is very stable (half-life 70 h at 37°C) and its spontaneous (and slow) breakdown proceeds via fragmentation of the penicilloyl group. It should be noted that heat denaturation of the complex, without any further proteolytic treatment, already destabilizes the complex, prevents the fragmentation reaction from occurring and, consequently, causes the release of benzylpenicilloate. With the R61 DD-carboxypeptidase, denaturation and proteolytic degradation of the benzylpenicilloyl–enzyme complex have also drastic effects on the stability of the complex and the nature of the released products (Frère *et al.*, 1976*a*; C. Duez, J. M. Frère, J. M. Ghuysen, J. Van Beeumen & J. Vandekerckhove, unpublished work). These observations can be best interpreted (Ghuysen *et al.*, 1979) by assuming that formation of an ester bond between C-7 of the penicillin molecule and the active serine residue on the enzyme (binding site 1) involves other interactions between both the acyl side chain and the monocyclic thiazolidine ring of the bound metabolite, and two specific amino acid groupings (enzyme binding sites 2 and 3). The relative disposition of these three enzyme sites (which depends on the conformation of the penicilloyl–enzyme complex) and, consequently, the distortion that they confer on the penicilloyl moiety, appear to be an important parameter that governs the stability of the complex and the fate of the bound metabolite.

A serine residue (70) is also involved in the interaction between penicillin and the β-lactamase I of *B. cereus* and that of *Escherichia coli* RTEM (Knott-Hunziker *et al.*, 1979; Fisher *et al.*, 1980; J. R. Knowles, personal communication), and this serine residue is conserved in two other β-lactamases of known sequences, i.e. those of *Staphylococcus aureus* and *B. licheniformis* (Ambler, 1979). It thus follows that the amino acid sequences around the

Table 1. *Sequences around the active serine residue in four* DD-*carboxypeptidases and four β-lactamases*
The active serine in the *B. subtilis* DD-carboxypeptidase occurs at position 36.

DD-Carboxypeptidases or DD-carboxypeptidases–transpeptidases				
Streptomyces R61	Val-Gly-	Ser		Frère *et al.*, 1976*a*
Actinomadura R39	Leu-Pro-Ala-	Ser	-Asn-Gly-Val	The present paper
B. stearothermophilus	Gly-Ile-Ala-	Ser	-Met	Yocum *et al.*, 1979
B. subtilis	Pro-Ile-Ala-	Ser	-Met-Thr-Lys	Yocum *et al.*, 1979
β-Lactamases				
Staphylococus aureus	Ala-Tyr-Ala-	Ser	-Thr-Ser-Lys	Ambler, 1979
B. cereus 569/H I	Ala-Phe-Ala-	Ser	-Thr-Tyr-Lys	Ambler, 1979
B. licheniformis 749/C	Ala-Phe-Ala-	Ser	-Thr-Ile-Lys	Ambler, 1979
E. coli RTEM	Pro-Met-Met-	Ser	-Thr-Phe-Lys	Ambler, 1979

presumed active serine residue are known for four different DD-carboxypeptidases and four different β-lactamases. Alignment of the active serine residues in these eight different enzymes (Table 1) shows an obvious homology only between the four β-lactamases, as well as between the two *Bacillus* DD-carboxypeptidases.

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