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OCCURRENCE OF A SERINE RESIDUE IN THE PENICILLIN-BINDING SITE OF THE EXOCELLULAR DD-CARBOXY-PEPTIDASE-TRANSPEPTIDASE FROM *STREPTOMYCES* R61

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1. Introduction

According to the hypothesis proposed by Tipper and Strominger in 1965 for the mechanism of action of penicillin [1], the antibiotic, acting as a structural analogue of the substrate, would acylate an essential sulfhydryl group of an enzyme that catalyzes peptide crosslinking during wall peptidoglycan synthesis in bacteria. However, the experimental results presented in favour of the involvement of a sulfhydryl group in the transpeptidation reaction as well as in the binding of penicillin have never been very convincing. The exocellular DD-carboxypeptidase-transpeptidase that is excreted by *Streptomyces* R61 during growth, is very sensitive to penicillin [2] and appears to be a good model for the study of the interaction between the enzyme, substrates and antibiotic [3,4]. At 37°C, the stoichiometric complex formed between benzylpenicillin and this enzyme decomposes with half-life of 80 min, yielding active enzyme, phenylacetylglycine and *N*-formyl-D-penicillamine [5,6]. More recently, however, it has been observed that when a solution of the [^{14}C]benzylpenicillin–enzyme complex (in 10 mM phosphate buffer, pH 7.0) is boiled for 1 min, the radioactivity remains stably attached to the denatured protein. Moreover, whereas the native enzyme is very resistant to the action of various proteases, the denatured protein can be readily degraded by trypsin or pronase. In the present report, we show that in the boiled radioactive benzylpenicillin–enzyme complex, the radioactivity is fixed to a serine residue.

2. Materials and methods

The R61 enzyme, the [^{14}C]benzylpenicillin (17 mCi/mmol; side-chain) and [^{3}H]benzylpenicillin (1.7 mCi/mmol; with the ^{3}H- on the β-methyl group of the thiazolidine moiety) were those previously used [5,6]. The benzylpenicillin–enzyme complex was formed and purified as described previously [7] (for more details, see legend of fig.1). In addition to the R61 DD-carboxypeptidase-transpeptidase, the following enzymes were also used: pronase (Sigma); trypsin (Worthington); thermolysin (Daiwa Kasei); leucine amino peptidase (Worthington); the amino peptidase from *Streptomyces albus* G [8]; carboxypeptidase A (Worthington) and carboxypeptidase B (Worthington). High-voltage electrophoreses were carried out with a Gilson High Voltage Electrophorator, model DW. The following chromatography solvents were used. I; 1-butanol/acetic acid/pyridine/water (15:3:10:12, v/v/v/v); II; chloroform/methanol/acetic acid (88:10:2, v/v/v); III; 1-butanol/water/conc. ammonia (100:97:3, v/v/v., upper phase).

3. Results

3.1. *Degradation of the boiled benzylpenicillin–R61-enzyme complex with pronase*

Treatment of the boiled [^{14}C]benzylpenicillin–enzyme complex with pronase for 30 min (see legend of figure 1) quantitatively yielded a radioactive, negatively charged peptide, (i.e. pronase peptide I) which exhibited a mobility of 10 cm/h by high voltage paper electrophoresis at neutral pH (fig.1A). Treatment to 90 min partially transformed this peptide into a smaller one, of higher mobility (pronase peptide II; fig.1B). Further treatment with a larger amount of pronase had no effect on this latter pronase peptide II. Peptides of identical electrophoretic mobilities were obtained when the [^{3}H]benzylpenicillin–R61-enzyme complex was degraded under the same conditions as above, thus demonstrating that both the benzyl side-chain and the thiazolidine moiety of the benzyl-penicillin molecule were attached to the peptides.

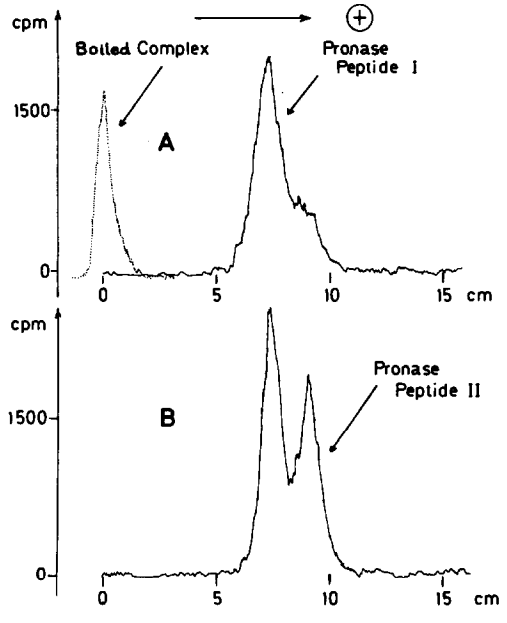


Fig.1. Detection of pronase peptides I and II by high-voltage paper electrophoresis. About 7.6 mg of enzyme (200 nmol) were treated by a 3-fold excess of [^{14}C]benzylpenicillin (17 mCi/nmol; Amersham) for 10 min at 37°C in 0.5 ml of 10 mM sodium phosphate buffer, pH 7.0. The complex was isolated by filtration through a short (1.5 × 10 cm) Sephadex G-25 column in the same buffer. Concentration of enzyme in the pooled fractions was 2.2 mg/ml. The solution was boiled for 1 min. A heavy protein precipitate was formed. A sample of the boiled complex (30 μl, i.e. 70 μg) was treated at 37°C with 6 μg of pronase (Sigma) in a total volume of 50 μl of 10 mM phosphate pH 7.0. After 0, 30 and 90 minutes, 4, 10 and 15 μl aliquots, respectively, were submitted to high-voltage paper electrophoresis (60 V/cm), at pH 6.5, for 45 min. The figure represents the radioactivity profiles on the electrophoretograms. A (dotted line): after 0 min (i.e. the original boiled penicillin–enzyme complex); A (solid line): after 30 min of degradation B: after 90 min of degradation. Scanning conditions: 2 cm/min, time constant: 10 s. Packard 2000 radiochromatogram scanner.

Pronase peptide I was prepared from 2.8 mg of boiled R61-enzyme–[^{14}C]benzylpenicillin complex (see legend of figure 1) by treatment with 200 μg of pronase for 20 min at 37°C in 3 ml 10 mM phosphate buffer pH 7.0. Electrophoresis of a 10 μl aliquot revealed the presence of pronase peptide I alone. The peptide was purified by gel filtration on a column of Sephadex G-25 (1.5 × 70 cm) in water, followed by high-voltage electrophoresis at pH 6.5, paper chromatography in solvent I (R_F = 0.65) and finally high-voltage electrophoresis at pH 3.5. At this latter pH value, the peptide was positively charged and exhibited a mobility of 7 cm/h (30 V/cm). The above gel filtration indicated a molecular weight of about 600–700.

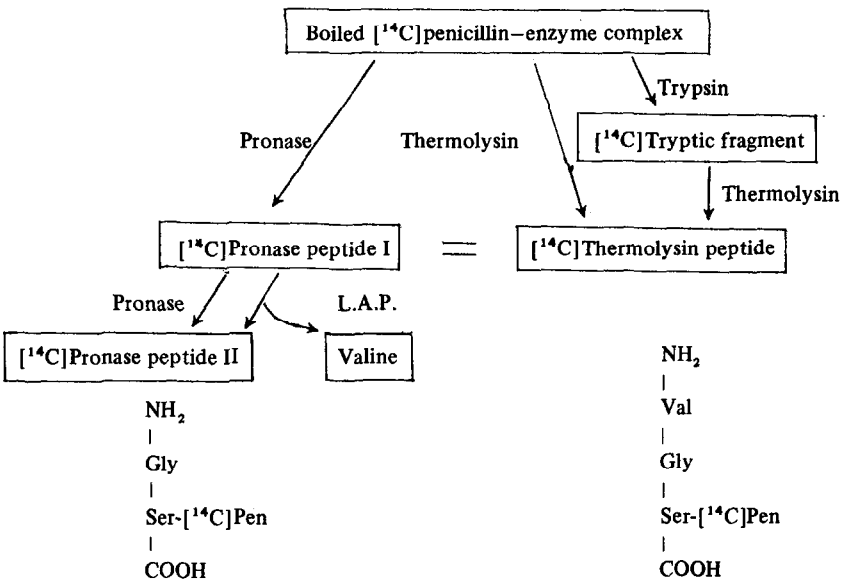
3.2. *Degradation of the boiled benzylpenicillin - R61 enzyme complex with trypsin*

Treatment of the boiled [^{14}C]benzylpenicillin–enzyme complex with trypsin (for conditions, see legend of scheme 1) yielded a radioactive compound which, after high-voltage electrophoresis at pH 6.5 stayed on the baseline suggesting that penicillin was bound to a protein fragment of considerable size.

3.3. *Degradation of the boiled benzylpenicillin–R61-enzyme complex with thermolysin*

Treatment of the boiled [^{14}C]benzylpenicillin–enzyme complex with thermolysin (for conditions, see legend of scheme 1) yielded a radioactive peptide (thermolysin peptide) exhibiting the same electrophoretic mobility as pronase peptide I. Treatment of the radioactive tryptic fragment with thermolysin also yielded the same peptide. The thermolysin peptide was purified as described above for the pronase peptide I except that the gel filtration was omitted. In all systems used, both the pronase peptide I and the thermolysin peptide have the same relative mobilities and R_F values.

Scheme 1
Degradation of the complex and proposed structures for the peptides



Conditions for the enzymatic degradations. Pronase: see legend of figure 1 and text. Trypsin: a 2 ml sample containing 4.4 mg of boiled R61-enzyme–[^{14}C]benzylpenicillin complex (see legend of figure 1) was dialysed against 0.5% ammonium bicarbonate and treated for 2 h at 37°C with 60 μg of trypsin. At the end of the incubation, a precipitate was still present in the reaction mixture. A sample (450 μg) was submitted to electrophoresis at pH 6.5; the radioactivity stayed on the base-line. Thermolysin: the bulk suspension after trypsin treatment was degraded with 60 μg of thermolysin for 3 h at 45°C in 0.2 M ammonium acetate buffer, pH 8.5, containing 5 mM $CaCl_2$. The solution became clear. The radioactive thermolysin peptide was purified by the same techniques as pronase peptide I. Direct degradation of the boiled R61-enzyme–[^{14}C]benzylpenicillin complex was performed under the same conditions. Leucine aminopeptidase (LAP): about 5 nmol of pronase peptide I were treated with 4 μg of Mn^{2+}-activated LAP [9] in 100 μl of 10 mM Tris–Cl buffer, pH 8.5 containing 6 mM $MgCl_2$.

3.4. *Composition of pronase peptide I and thermolysin peptide*

Acid hydrolysis (6 N HCl; 105°C; 20 h) yielded from both peptides equimolar amounts of serine, glycine and valine (actual ratios 0.9:1.0:0.8 for pronase peptide I and 1.1:1.0:0.6 for thermolysin peptide) which on the basis of the radioactivity of the original sample, corresponded to the amount of peptides hydrolyzed. Smaller, contaminating amounts of aspartic acid, threonine, glutamic acid and alanine were also found. By using the dansyl-Edman technique, the sequence of the thermolysin peptide was established as H-Val-Gly-Ser-OH. The radioactivity was lost after the first step of the degradation probably during the treatment with trifluoroacetic acid. Treatment of pronase peptide I with leucine aminopeptidase (see legend of scheme 1) gave rise to a [^{14}C]peptide exhibiting the same mobility as pronase peptide II. Valine was released during the process and glycine was then identified as the N-terminal amino acid of the [^{14}C]dipeptide by dinitrophenylation, HCl hydrolysis and two-dimensional chromatography of the ether extract of the hydrolysate (first in solvent II and then in solvent III). Further treatment of pronase peptide II with leucine aminopeptidase was without effect. Similarly, the aminopeptidase from *Streptomyces albus* G and both carboxypeptidases A and B had no effect neither on pronase peptide I nor on pronase peptide II.

3.5. *Degradation of the 1-butanol denatured benzylpenicillin–R61-enzyme complex with pronase*

As denaturation by boiling may seem to be a very drastic procedure, a milder technique was used. To a solution of the R61-enzyme–[^{14}C]benzylpenicillin complex (1 mg in 0.5 ml of 10 mM phosphate buffer pH 7.0), 1 ml of 1-butanol was added. After vigorous shaking at 20°C, the two phases were allowed to separate and a layer of denatured protein was formed at the interface. Pronase (100 μg) was added to the lower phase and after incubation at 37°C for 30 min, the denatured protein was solubilized and a peptide exhibiting all the properties of pronase peptide I was formed. Only 50% of the original radioactivity, however, was recovered in the peptide. Part of the missing radioactivity occurred as native complex and the other part as phenylacetylglycine, thus indicating that denaturation had not been complete.

4. Conclusions

From the foregoing it follows that penicillin can only be bound to the carboxyl-terminal serine residue of the isolated peptides. Three hypotheses can be proposed to explain these results: (1) the penicillin molecule is bound to the serine residue in the native complex; (2) the penicillin molecule is bound to another residue and is transferred on the serine residue during denaturation; and (3) the penicillin molecule is not bound covalently to any residue and binding to the serine residue occurs upon denaturation. The fact, however, that boiling and 1-butanol treatment of the R61-enzyme–benzylpenicillin complex lead to the same results strongly suggests the existence of a covalent serine–penicillin bond in the original enzyme–antibiotic complex. Fixation on the hydroxyl group of serine would be most likely. Consequently, the degradation of penicillin into *N*-acylglycine and *N*-formyl-D-penicillamine, that is catalyzed by the native enzyme, may be a very slow enzyme process. When the tertiary architecture of the enzyme is disrupted by denaturation, this degradation no longer takes place. One should note, however, that phenylmethanesulfonyl fluoride, a specific inhibitor of the serine proteases, had no effect on the activity of the enzyme nor on its ability to bind penicillin (at least after incubation of the enzyme with 1 mM phenylmethanesulfonyl fluoride in 10 mM sodium phosphate buffer, pH 7.0, at 37°C for 60 min). With chymotrypsin, trypsin and acetylcholinesterase, the second order rate constant values for the inactivation are 14, 900, 271 and < 0.06 M^{-1} min^{-1}, respectively [10].

Acknowledgements

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