Conversion of isopenicillin N into penicillin N in cell-free extracts of *Cephalosporium acremonium*

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In a cell-free system prepared by osmotic lysis of protoplasts of *Cephalosporium acremonium*, isopenicillin N is converted into penicillin N. The epimerase activity of the system is labile.

It has now been established that the tripeptide δ -(L-aminoadipyl)-L-cysteinyl-D-valine is incorporated intact into a penicillin molecule in a crude cell-free system prepared by lysis of protoplasts of *Cephalosporium acremonium* (Fawcett *et al.*, 1976), and that the first penicillin formed is isopenicillin N [I; RCO = δ -L- α -aminoadipyl] (O'Sullivan *et al.*, 1979; Konomi *et al.*, 1979). It has also been shown that penicillin N [II; RCO = δ -(D- α -aminoadipyl)] is a precursor of deacetoxycephalosporin C [II; RCO = δ -(D- α -aminoadipyl)] (Baldwin *et al.*, 1980) in *C. acremonium*. Since intact cells of this fungus excrete penicillin N (Abraham *et al.*, 1954), the latter is presumably formed by epimerization of the L- α -aminoadipyl side chain of isopenicillin N. The present paper reports evidence for the presence of an epimerase that catalyses this reaction in cell-free extracts of *C. acremonium*. The existence of such an enzyme has been briefly reported by Abraham *et al.* (1980).

Experimental

Materials

Potassium cephalosporin C and a lytic enzyme preparation L1 from *Cytophaga* sp. were kindly provided by Glaxo Group Research, Greenford, Middx., U.K. Sodium penicillin N was a laboratory sample purified by the method of Smith *et al.* (1967) to give a product that was about 80% pure. Potassium isopenicillin N (about 90% pure) was synthesized by the method of Usher *et al.* (1975).

Other reagents (AnalaR grade where available) were from BDH Chemicals, Poole, Dorset, U.K.

Methods

(a) The preparation of protoplasts of *C. acremonium* from mycelium normally harvested after 48 h was carried out as described by Fawcett *et al.* (1976) by use of a lytic enzyme preparation L1 from *Cytophaga*. For the preparation of a cell-free system by osmotic lysis of the protoplasts the method of Fawcett *et al.* (1976) was also used, except that the buffer was 50 mM-sodium 4-morpholinepropanesulphonate (pH 7.0). For some experiments the crude protoplast lysate was centrifuged at 50 000g for 10 min at 4°C. The supernatant was removed and the sediment resuspended in an equal volume of buffer. The protein concentrations of the whole cell-free system and the 50 000g supernatant were determined by the method of Bradford (1976) with bovine serum albumin as a standard.

A few experiments were carried out with intact protoplasts suspended in 0.8 M-NaCl solution.

(b) Incubation of the preparation of potassium isopenicillin N (100 µg/ml) in the crude cell-free system (0.5 ml) was carried out at 27°C for up to 3 h in stoppered 0.75 ml reaction vessels on a rotary shaker (250 rev./min).

(c) The production of penicillin N from isopenicillin N could be followed by antibacterial assay, since the preparation of isopenicillin N showed a much lower activity than penicillin N against *Salmonella typhi* (strain 'Mrs. S'; Felix & Pitt, 1935), although not against *Staphylococcus aureus* N.C.T.C. 6571 (Usher *et al.*, 1975). In the present experiments the quotient (activity of isopenicillin N)/(activity of penicillin N) was found to be 0.04 for *Salm. typhi* and 0.68 for *Staph. aureus*.

Solutions were assayed in duplicate by the hole-plate method (Smith *et al.*, 1967). Pure potassium cephalosporin C, which was arbitrarily assigned an activity of 10 units/mg, was used in

three concentrations (6, 3 and 1.5 units/ml respectively) to provide a standard log concentration-response line. The lowest activity against *Salm. typhi* detectable under these conditions varied from 0.35 to 0.7 units/ml with different batches of assay plates and was determined for each experiment. The activity of pure penicillin N against *Salm. typhi* was taken to be equivalent to 100 units of cephalosporin C/mg in accordance with the value given by Smith *et al.* (1967).

Samples were removed from the incubation mixture at intervals of 0.5 h and cooled in ice. All the samples were then assayed against *Salm. typhi*. In the experiments described the protoplast lysate alone produced no detectable zone of inhibition, nor did the preparation of isopenicillin N alone in the initial concentration in which it was used (100 µg/ml of material 90% pure; 0.36 units/ml), although a zone was just detectable when the concentration was raised to 125 µg/ml (0.45 units/ml). In contrast, the preparation of penicillin N (80% pure) produced a just detectable zone at 5 µg/ml (0.4 units/ml). In some experiments the reaction mixtures were treated with β -lactamase I from *Bacillus cereus* 569/H/9 (Davies *et al.*, 1974) for 10 min at room temperature before assay. The amount (5 µl) of the solution of β -lactamase I added was sufficient to destroy the penicillins in the mixtures.

Although the activity of penicillin N against *Staph. aureus* was significantly greater than that of isopenicillin N the difference in activity was not sufficient for the organism to be used to follow the formation of penicillin N from isopenicillin N.

Results and discussion

The addition of isopenicillin N to lysates of protoplasts of *C. acremonium* to give an initial concentration of 90 µg/ml resulted in the generation of activity against *Salm. typhi* when the mixture was incubated at 27°C. This activity was destroyed by purified β -lactamase I (Davies *et al.*, 1974) and could therefore be attributed to a penicillin. No activity was detected when the lysate used had been heated at 100°C for 5 min. These findings indicated that an epimerase in the incubation mixture catalysed the formation of penicillin N from isopenicillin N. When a suspension of intact protoplasts was used in place of the lysate no production of penicillin N was observed. Hence the epimerase is apparently not located on the outside of the protoplast membrane.

As reflected in the amounts of penicillin N formed after 2 hours the epimerase activities of lysates of protoplasts prepared from equal weights of damp-dry mycelium harvested from the same fermentation at 36 h and 60 h were about 15% and 50% respectively of those obtained when harvesting was at 48 h. However, the protein concentrations of these

three lysates were 15.7, 24 and 7.25 mg/ml respectively and there were considerable variations in the activities and protein concentrations of lysates from batches of mycelia harvested at 48 h from different fermentations. Storage of harvested mycelium at -20°C for 3 days before use did not appear to diminish the activity of the lysate obtained.

The formation of penicillin N from isopenicillin N in two protoplast lysates is shown in Fig. 1. Although the activity of isopenicillin N present initially (0.36 units/ml) was too low to give a detectable zone of inhibition, its small and decreasing contribution to the activities measured as the reaction proceeded were allowed for in the following calculation: penicillin N (µg/ml) = [(units/ml) - 0.36]/0.096. A further experiment showed that most of the epimerase activity was present in the supernatant after centrifugation of the lysate at 50000g. After incubation for 2 h, 13.0 µg of penicillin N/ml and 3.1 µg of penicillin N/ml had been

Fig. 1. Conversion of isopenicillin N into penicillin N. Isopenicillin N (90 µg/ml) was incubated at 27°C with 0.5 ml of protoplast lysates (I and II) prepared from mycelium harvested at 48 h from two different fermentations. The protein concentrations of lysates I and II were 7.3 and 29 mg/ml respectively. The amounts of penicillin N formed were calculated as described in the text from antibacterial assays with *Salm. typhi* as the test organism.

formed in the supernatant (16.25 mg of protein/ml) and the corresponding suspension of the sediment (4.5 mg of protein/ml) respectively.

The epimerase in the cell-free system was unstable. In contrast with its relative stability in mycelial cells at -20°C only about 15% of its original activity survived storage of the crude cell-free extracts at -20°C or at 4°C for 24 h. Rapid freezing of the system followed by immediate thawing decreased its activity by 50%. Addition to the protoplast lysate of phenylmethanesulphonyl fluoride, and EDTA (1 mM final concentrations) as potential inhibitors of proteolysis did not enhance epimerase stability and its activity was not stimulated by 1 mM-dithiothreitol.

When penicillin N, instead of isopenicillin N, was added to the protoplast lysate to give a final concentration of $10\mu\text{g/ml}$ and the mixture incubated at 27°C for 2 h, there was no detectable decrease in the activity of the mixture against *Salm. typhi*. However, since a decrease of less than 10% might not have been apparent, the assay procedure used was inadequate for a study of the reversibility of the epimerization of isopenicillin N to penicillin N.

O'Sullivan *et al.* (1979) suggested that the penicillin N obtained from fermentations of *C. acremonium* is formed by epimerization of the side chain of isopenicillin N and Konomi *et al.* (1979) postulated that this reaction is brought about by a labile enzyme. Baldwin *et al.* (1981) have shown that isopenicillin N is converted into a substance that behaves like deacetoxycephalosporin C in a cell-free system from protoplasts of *C. acremonium* mutant M-0198, but that a component of this system is unstable at -80°C . Since the ability of the system to convert penicillin N into deacetoxycephalosporin C was retained after storage at -20°C they inferred that isopenicillin N is converted into penicillin N by a labile epimerase. Direct evidence for the presence

of such an enzyme is given in the present paper. No significant ring expansion of penicillin N to deacetoxycephalosporin occurred under the conditions used, presumably because Fe(II), ascorbic acid and ATP are needed for this reaction (Baldwin *et al.*, 1980) and were not added to the system.

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