The Conversion of Cephalosporins to 7 α -Methoxycephalosporins by Cell-Free Extracts of *Streptomyces clavuligerus*

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(Received 12 November 1979)

In the presence of *S*-adenosylmethionine, 2-oxoglutarate, Fe²⁺ and a reducing agent, cell-free extracts of *Streptomyces clavuligerus* convert cephalosporin C and *O*-carbamoyldeacetylcephalosporin C into 7 α -methoxy derivatives. No synthesis of a 7 α -methoxy derivative of deacetylcephalosporin C was detected in the system used, and the 7 α -methoxy derivative of deacetoxycephalosporin C was produced only in relatively small amounts. It appears that the 7 α -methoxy group is introduced after the cephalosporin ring system has been formed and that its introduction may represent the final step in a biosynthetic pathway.

Certain species of *Streptomyces* produce penicillin N and various cephalosporins with a δ -(α -aminoadipyl) side chain (Nagarajan *et al.*, 1971; Stapley *et al.*, 1972). *S. clavuligerus* produces the *O*-carbamoyl derivative of deacetylcephalosporin C (compound I; X = H, R = OCONH₂) and its 7 α -methoxy analogue, cephamycin C (compound I; X = OMe, R = OCONH₂). Cysteine, valine and α -aminoadipic acid are precursors of cephamycin C and the methyl moiety of the 7 α -methoxy group of this cephalosporin is derived from methionine (Whitney *et al.*, 1972).

O'Sullivan *et al.* (1979a) showed that the oxygen in the 7 α -methoxy group of cephamycin C comes from molecular oxygen. The experiments described here were designed to throw light on the stage in the biosynthetic pathway at which the methoxy group is introduced.

Materials and Methods

Materials

DL-[1-¹⁴C]Valine (37.5 mCi/mmol), L-[Me-³H]-methionine (100 mCi/mmol) and *S*-adenosyl-L-[Me-³H]methionine (15 Ci/mmol) were supplied by The Radiochemical Centre, Amersham, Bucks., U.K. The labelled *S*-adenosylmethionine was diluted with

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the corresponding unlabelled compound before use. Cefuroxime and cephalosporin C were kindly provided by Glaxo Group Research Ltd., Greenford, Middx., U.K.; cephalothin, 7-methoxycephalosporin C, deacetoxycephalosporin C and the *O*-carbamoyl derivative of deacetylcephalosporin C by Eli Lilly and Co., Indianapolis, IN, U.S.A.; clavulanic acid by Beecham Pharmaceuticals, Brockham Park, Surrey, U.K.; nocardicin A by Fujisawa Pharmaceutical Co., Osaka, Japan; and cephamycin C by Merck, Sharp and Dohme Research Laboratories, Rahway, NJ, U.S.A. Deacetylcephalosporin C was prepared by the method of Jeffrey *et al.* (1961). Other reagents (AnalaR grade where available) were from BDH Chemicals, Poole, Dorset, U.K.

Methods

(a) Culture and growth conditions of *S. clavuligerus* were as described by O'Sullivan *et al.* (1979a).

(b) For the preparation of cell-free extracts, mycelium was harvested after 24 h growth in 50 ml of medium by centrifugation (6000 *g* for 5 min) and washed by resuspension in water and centrifugation as just described. The mycelium was pressed between filter papers and the damp-dry material (1.5 g) was suspended in a 20 ml-volume centrifuge tube (7.5 cm long $\times$ 2.0 cm diam.) in 4 ml of Mops (4-morpholinepropanesulphonic acid) buffer (0.01 M) adjusted to pH 7.5 with 1 M-NaOH. The suspension was cooled on ice and subjected to ultrasonic treatment in an MSE ultrasonicator (60 W, 20 kHz) for four periods of 15 s with 1 min intervals, the titanium probe (9 mm diam.) being submerged about

1 cm below the surface of the liquid. The mixture was then centrifuged at 25000g for 30 min. The supernatant (about 5 ml) was dialysed at 4°C for 5–17 h against water (5 litres) before use as the cell-free enzyme solution. The protein concentration of this solution, determined by the method of Bradford (1976), with bovine serum albumin as a standard, was about 10 mg/ml.

(c) The reaction in the cell-free system was carried out at 30°C for 1 h. The reaction mixture contained a cephalosporin substrate (cephalosporin C or *O*-carbamoyldeacetylcephalosporin C), 2-oxoglutarate and L-ascorbate (both added as their free acids), dithiothreitol and FeSO₄ [all 1 mM (final concn.)], Mops buffer (25 mM), adjusted to pH 7.5 with 1 M-NaOH, *S*-adenosyl[Me-³H]methionine (0.7 μCi; 36 mCi/mmol) and 0.2–0.5 ml of enzyme solution. The final volume of the mixture was 1 ml. In similar control experiments, no cephalosporin was added. The reaction was stopped by the addition of 50 μl of acetic acid, the mixture was centrifuged and 0.8 ml of the supernatant applied to a column (2 cm × 0.6 cm) of Dowex 1 resin (X8; acetate form; 200–400 mesh). The column was washed with water (15 ml) to remove unchanged *S*-adenosyl[Me-³H]methionine, and radioactive 7-methoxycephalosporin was then eluted with 1 M-(NH₄)₂SO₄ (4 ml). The radioactivity in 0.2 ml of eluate was measured, after the addition of 3 ml of water-miscible scintillation fluid (Bruno & Christian, 1961), in a Nuclear-Chicago Unilux 11A liquid-scintillation counter. The counting efficiency was 23%. The identity of the labelled product in the eluate was determined by comparison of its behaviour with that of authentic cephamycin C or 7α-methoxycephalosporin C on paper electrophoresis at pH 6.5 (70 V/cm) followed by chromatography as previously described by O'Sullivan *et al.* (1979a). Radioactive spots were revealed by fluorography (Laskey & Mills, 1975) under the conditions used by O'Sullivan *et al.* (1979b). In some cases segments of the paper (1 cm × 3 cm) after electrophoresis were counted in the scintillation counter after immersion in 3 ml of the water-miscible scintillation fluid. In this procedure the counting efficiencies for ³H and ¹⁴C were about 15 and 45% respectively.

A similar procedure was used when deacetylcephalosporin C, deacetoxycephalosporin C, cephalothin, cefuroxime, penicillin N, nocardicin A and clavulanic acid were tested as substrates.

(d) To determine whether peptide precursors were labelled from [Me-³H]methionine, damp-dry mycelium (20 g, harvested after 48 h) was resuspended in water (85 ml) in a 1-litre conical flask with four indentations (9 cm high × 1.5 cm wide) in its walls, and shaken at 160 rev./min at 27°C. L-[Me-³H]-Methionine (50 μCi) or DL-[1-¹⁴C]valine (15 μCi) was added, and samples (0.3 ml) were removed at

intervals. After centrifugation of the samples (6000g for 15 s) the radioactivities of the supernatant were measured. Mycelium for peptide analysis was harvested by centrifugation (6000g for 5 min) after 1 h, when the net uptake of radioactivity from the labelled amino acids had ceased and radioactive cephamycin C began to appear in the extracellular fluid.

The mycelium was mixed with 40 ml of 7.5% (w/v) trichloroacetic acid in a Waring blender for 30 s. After the mixture had been left at room temperature for 30 min, it was filtered and washed with 20 ml of 5% (w/v) trichloroacetic acid. The washings and filtrate were combined and extracted three times with an equal volume of diethyl ether. The diethyl ether was removed *in vacuo* from the aqueous phase and the latter was freeze-dried. The residue was suspended in 1 ml of water and applied to a column (4 cm × 1 cm) of Dowex 50 resin (X4; H⁺ form; 200–400 mesh). Peptides were eluted with 1 M-pyridine and the eluate freeze-dried. After oxidation of the residue with performic acid (Smith *et al.*, 1967) the peptide sulphonic acids were separated from other compounds by electrophoresis on paper at pH 1.8 for 2.5 h, followed by chromatography in butan-1-ol/acetic acid/water (4:1:4, by vol.; upper phase) and analysed by fluorography.

In a separate experiment, with 100 g of damp-dry mycelium harvested after 48 h, the peptide sulphonic acids and penicillaminic acid were revealed by a ninhydrin spray after electrophoresis and chromatography on paper. The intensity of the spot corresponding to δ-(L-α-aminoadipyl)-L-cysteicyl-D-valine indicated that about 45 nmol of the cysteinyl tripeptide from which it was derived was present in 20 g of the mycelium. The corresponding amount of penicillaminic acid was 5–10 nmol.

The extracellular fluids obtained after centrifugation of mycelium that had been resuspended in the presence of radioactive valine and methionine respectively were freeze-dried and the products analysed by electrophoresis on paper at pH 6.5. The radioactivity in the position corresponding to cephamycin C was determined as described above.

Results

Biosynthesis of cephamycin C and its peptide precursor in suspensions of S. clavuligerus

Analysis of the peptide sulphonic acids and penicillaminic acid in mycelium that had been resuspended in water and shaken in the presence of [1-¹⁴C]valine (15 μCi) for 1 h showed that ¹⁴C was incorporated into δ-(α-aminoadipyl)cysteicyl-valine (6 nCi) and into penicillaminic acid (presumably derived from penicillin N) to a much smaller extent (about 0.3 nCi). The latter value was near to

the limit of detection (about 0.15 nCi) of a ^{14}C -labelled compound on paper by fluorography. With a corresponding sample of mycelium shaken in the presence of [$\text{Me-}^3\text{H}$]methionine (50 μCi) there was no evidence of ^3H in any peptide sulphonic acid or in penicillaminic acid, the limit of detection being about 0.5 nCi. In contrast, the extracellular cephamycin C produced in the presence of DL-[1- ^{14}C]valine and L-[$\text{Me-}^3\text{H}$]methionine was labelled with ^{14}C (80 nCi, 0.5% of the added radioactivity) and ^3H (1.27 μCi , 2.5% of the added radioactivity) respectively. In three separate experiments in which damp-dry mycelium (5 g) was resuspended in water (50 ml) in the presence of [^{14}C]valine for a longer period (about 6 h), the specific radioactivities of the extracellular cephamycin C formed (about 3 mg) were respectively 1.12, 0.43 and 2.3 mCi/mmol.

Biosynthesis of 7 α -methoxycephalosporins in a cell-free system

When cephalosporin C (compound I; X = H, R = OCOCH_3) was used as a substrate in the cell-free system, the incorporation of ^3H from *S*-adenosyl-L-[$\text{Me-}^3\text{H}$]methionine into 7 α -methoxycephalosporin C (I; X = OMe, R = OCOCH_3) increased linearly with an increase in the protein concentration in the system from 1 to 4 mg/ml. It also increased linearly with time for 1 h when the concentration of protein was 4 mg/ml. When the pH of the reaction mixture was varied (from 6.5 to 8.0 in 50 mM-Mops with NaOH, and from 7.5 to 9.0 in 50 mM-Tris with HCl) the incorporation of ^3H was found to be maximal at pH 7.5. When Fe^{2+} or 2-oxoglutarate was omitted from the system, no incorporation of ^3H into 7 α -methoxycephalosporin C was detected. The omission of dithiothreitol appeared to have no effect, but in the absence of ascorbate the incorporation was decreased (Table 1).

With *O*-carbamoyldeacetylcephalosporin C as a substrate, ^3H was incorporated into cephamycin C. In this case, also, Fe^{2+} , 2-oxoglutarate and ascorbate were required for maximum incorporation, which was similar to that into 7 α -methoxycephalosporin C when cephalosporin C was the substrate. With deacetoxycephalosporin C (I; X = H, R = H) as substrate, there was a relatively small incorporation of ^3H (14% of the amount with cephalosporin C) into a compound whose behaviour on paper electrophoresis and chromatography was that to be expected for 7 α -methoxydeacetoxycephalosporin C. The addition to the system of deacetoxycephalosporin C, together with cephalosporin C (both 1 mM) resulted in a small but reproducible inhibition (5–10%) of the incorporation observed when cephalosporin C was used alone.

No formation of a 7-methoxycephalosporin from deacetylcephalosporin C (I; X = H, R = OH) was observed in this enzyme system. Cefuroxime and cephalothin, with an *O*-carbamoyl and *O*-acetyl group respectively at C-3', but with *N*-acyl side chains different from the δ -(D- α -aminoadipyl) side chain of the natural cephalosporins, also failed to yield 7-methoxy derivatives in detectable quantities, and no evidence was obtained for the incorporation of a methoxyl group into penicillin N, clavulanic acid or nocardicin A.

Discussion

The failure to find a derivative of δ -(L- α -aminoadipyl)-L-cysteinyl-D-valine that had incorporated ^3H from *methyl*-labelled methionine in the mycelium of *Streptomyces clavuligerus* suggested that the methoxy group of 7 α -methoxycephalosporins was introduced after the cephalosporin ring system had been formed. The results described here with a cell-free system indicate that an enzyme required for

Table 1. *Requirements for the introduction of a 7 α -methoxy group into cephalosporin C in a cell-free system*
The reaction mixture (1 ml) contained 3 mg of protein and 0.7 μCi (19.4 nmol) of *S*-adenosyl-L-[$\text{Me-}^3\text{H}$]methionine. Cephalosporin C (potassium salt) and other reagents were all at 1 mM. The reaction was terminated after 1 h.

Reaction mixture	7 α -Methoxycephalosporin C formed		Relative incorporation of ^3H (%)
	(nCi)	(nmol)	
Complete	50.4	1.4 ± 0.04 (3)	100
Without			
Cephalosporin C	<1.5	<0.05	<3
2-Oxoglutarate	<1.5	<0.05	<3
FeSO_4	<1.5	<0.05	<3
Dithiothreitol	51.22	1.42 ± 0.03 (3)	102
L-Ascorbic acid	43.9	1.22 ± 0.04 (3)	87

the reaction has a relatively high substrate specificity and catalyses the final step in a biosynthetic pathway.

In its requirement of 2-oxoglutarate, Fe^{2+} and a reducing agent for maximum activity, one enzyme in *S. clavuligerus* that is involved in the introduction of a 7 α -methoxy group into *O*-carbamoyldeacetylcephalosporin C (and can also use cephalosporin C as a substrate) has properties of a dioxygenase (Abbott & Udenfriend, 1974). In this respect it resembles the enzyme that converts deacetoxycephalosporin C into deacetylcephalosporin C (Turner *et al.*, 1978). Deacetylcephalosporin C is the immediate precursor of cephalosporin C itself in *Cephalosporium acremonium* (Fujisawa *et al.*, 1975) and probably of *O*-carbamoyldeacetylcephalosporin C in *S. clavuligerus* (Brewer *et al.*, 1977).

The findings reported here suggest that the 7 α -methoxy group originates from an oxygenation of certain cephalosporins at C-7 and methylation of a hydroxylated intermediate by a methyltransferase. They are consistent with a previous conclusion of Whitney *et al.* (1972), based on the incorporation of ^{14}C and ^3H from 3,3'-labelled cystine residues into a 7-methoxycephalosporin, that the introduction of the methoxy group does not involve the formation of a double bond between the cysteine carbon atoms that become C-6 and C-7 respectively of the cephalosporins.

We thank the National Research Development Corporation and the Medical Research Council for financial assistance.

References

- Abbott, M. T. & Udenfriend, S. (1974) in *Molecular Mechanisms of Oxygen Activation* (Hayaishi, O., ed.), pp. 167–214, Academic Press, London and New York.
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.
- Brewer, S. J., Boyle, T. T. & Turner, M. K. (1977) *Biochem. Soc. Trans.* **5**, 1026–1029.
- Bruno, G. A. & Christian, J. E. (1961) *Anal. Chem.* **33**, 1216–1218.
- Fujisawa, Y., Shirafuji, H., Kida, M., Nara, K., Yoneda, M. & Kanzaki, T. (1975) *Agric. Biol. Chem.* **39**, 1295–1301.
- Jeffery, J. D'A., Abraham, E. P. & Newton, G. G. F. (1961) *Biochem. J.* **81**, 591–596.
- Laskey, R. A. & Mills, A. D. (1975) *Eur. J. Biochem.* **56**, 335–341.
- Nagarajan, R., Boeck, L. D., Gorman, M., Hamill, R. G., Higgins, C. E., Hoehn, M. M., Stark, W. M. & Whitney, J. G. (1971) *J. Am. Chem. Soc.* **93**, 2308–2310.
- O'Sullivan, J., Aplin, R. T., Stevens, C. M. & Abraham, E. P. (1979a) *Biochem. J.* **179**, 47–52.
- O'Sullivan, J., Bleaney, R. C., Huddleston, J. A. & Abraham, E. P. (1979b) *Biochem. J.* **184**, 421–426.
- Smith, B., Warren, S. C., Newton, G. G. F. & Abraham, E. P. (1967) *Biochem. J.* **103**, 877–890.
- Stapley, E. O., Jackson, M., Hernandez, S., Zimmerman, S. B., Currie, S. A., Mochales, S., Mata, J. M., Woodruff, H. B. & Hendlin, D. (1972) *Antimicrob. Agents Chemother.* **2**, 122–131.
- Turner, M. K., Farthing, J. E. & Brewer, S. J. (1978) *Biochem. J.* **173**, 839–850.
- Whitney, J. G., Brannon, D. R., Mabe, J. A. & Wicker, K. J. (1972) *Antimicrob. Agents Chemother.* **1**, 247–251.