The Oxygenation of [3-methyl-³H]Desacetoxycephalosporin C [7β-(5-D-Aminoadipamido)-3-methylceph-3-em-4-carboxylic acid] to [3-hydroxymethyl-³H]Desacetylcephalosporin C by 2-Oxoglutarate-Linked Dioxygenases from *Acremonium chrysogenum* and *Streptomyces clavuligerus*

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Cell-free extracts of *Acremonium chrysogenum* and *Streptomyces clavuligerus* oxidize the 3-methyl group of desacetoxycephalosporin C to a 3-hydroxymethyl group. The enzyme responsible for this reaction in these organisms was purified 20- and 30-fold respectively by chromatography on DEAE-cellulose. The enzymes, which were assayed with [3-methyl-³H]desacetoxycephalosporin C as substrate, have the properties expected of 2-oxoglutarate-linked dioxygenases. They require 2-oxoglutarate, Fe²⁺ cations and a mixture of reducing agents (dithiothreitol and ascorbate) for full activity. The enzyme from *A. chrysogenum*, but not that from *S. clavuligerus*, is activated about 10-fold when it is pre-incubated with a reaction mixture from which either desacetoxycephalosporin C or 2-oxoglutarate is omitted. Fe²⁺ cations seem to play a key role in this activation. Both enzymes seem highly specific for cephalosporins with the natural 7β-(5-D-aminoadipamido) side chain and are likely to be responsible for the oxidation of the 3-methylcephem nucleus *in vivo*.

Micro-organisms synthesize penicillins (Arnstein & Morris, 1960; Fawcett *et al.*, 1976) and cephalosporins (Loder & Abraham, 1971) from a tripeptide precursor that loses two or three pairs of hydrogen atoms during its oxidation to the 2,2-dimethylpenam or the 3-methylcephem nucleus respectively (Scheme 1). However, the final stage in the oxidation of the cephem nucleus involves the introduction of an oxygen atom. The product is a 3-hydroxymethylcephem, which the micro-organisms later modify, for example by acetylation (cephalosporin C) or by carbamoylation [7β-(5-D-aminoadipamido)-3-carbamoyloxymethylceph-3-em-4-carboxylic acid; see Brannon *et al.* (1972)].

The cephem ring is closed before the introduction of the oxygen atom. Desacetoxycephalosporin C, which has a 3-methylcephem nucleus, has been isolated from a number of cephalosporin producers (Kitano *et al.*, 1974; Higgins *et al.*, 1974) and it accumulates in some mutants of *Acremonium chrysogenum* that are unable to produce desacetylcephalosporin C (Fujisawa *et al.*, 1975). Furthermore, the isolation of an aldehyde almost certainly derived from a cephem-3-carbaldehyde (Fujisawa & Kanzaki, 1975) suggests that oxidation can proceed beyond the formation of a 3-hydroxymethylcephem.

The oxygen atom in the 3-hydroxymethyl group is derived directly from molecular O₂ (Stevens *et al.*, 1975), suggesting that the enzyme responsible for the oxidation of the 3-methyl group is an oxygenase.

Indeed indirect assays have suggested that a mono-oxygenase is involved in the oxidation of desacetoxycephalosporin C by cell-free extracts of *A. chrysogenum* (Liersch *et al.*, 1976; Fujisawa *et al.*, 1977). We now show, by a direct assay method, that the enzyme responsible in *A. chrysogenum* and in *Streptomyces clavuligerus* is, in fact, a 2-oxoglutarate-linked dioxygenase similar to enzymes such as thymine 7-hydroxylase and proline hydroxylase (Abbott & Udenfriend, 1974). A preliminary report of the work with *A. chrysogenum* has already been published (Brewer *et al.*, 1977b).

Materials

Cephalosporin C, desacetylcephalosporin C, desacetoxycephalosporin C, 7-aminocephalosporanic acid, 7-aminodesacetoxycephalosporanic acid, the diphenylmethyl ester of 7-aminodesacetoxycephalosporanic acid and other reagents used in the synthesis of cephalosporins were obtained from Glaxo Laboratories, Greenford, Middlesex, U.K. 1,4-Piperazinediethanesulphonic acid (Pipes) and dithiothreitol were obtained from BDH Chemicals, Poole, Dorset, U.K. Reduced glutathione and 5-(biphenyl-4-yl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole were obtained from Koch-Light, Colnbrook, Bucks., U.K. and NADH from Sigma Chemical Co., Kingston upon Thames, Surrey, U.K. Triton X-100 was obtained from Rohm and Haas, Croydon,

Scheme 1. Last stages in the biosynthesis of cephalosporins

Surrey, U.K. NE250 liquid scintillant was obtained from Nuclear Enterprises, Sighthill, Edinburgh, U.K.

The DEAE-cellulose preparations DE-23 and DE-32 were obtained from Whatman, Maidstone, Kent, U.K. The ultrafiltration membranes, PM10, were obtained from Amicon, High Wycombe, Bucks., U.K. Bio-Gel P2 and Dowex AG1 (200–400 mesh; X8) acetate were obtained from Bio-Rad Laboratories, Watford, Herts., U.K.

[3-methyl-³H]Desacetoxycephalosporin C and [3-methyl-³H]-7-aminodesacetoxycephalosporanic acid

[3-methyl-³H]Desacetoxycephalosporin C was prepared as described by Brewer *et al.* (1977b). 7-Amino[3-methyl-³H]desacetoxycephalosporanic acid was prepared at The Radiochemical Centre, Amersham, Bucks., U.K. by a similar method starting with 1 mmol of 7-aminocephalosporanic acid and 25 Ci of ³H₂ (about 10 Ci/mmol). The 7-aminocephalosporanic acid in 1 M-Na₂CO₃ (6 ml) was reduced by

the ³H₂ gas at room temperature for 15 min by using PdO (0.58 mmol) catalyst on kieselguhr. KOH pellets were used in a side arm to remove CO₂ produced in the reaction. The resulting mixture was acidified with 1 M-HCl (8.4 ml) and filtered on kieselguhr. The filtrate was decolorized with charcoal and adjusted to pH 6.0 with aq. NH₃.

The 7-amino[3-methyl-³H]desacetoxycephalosporanic acid formed was desalted by eluting it with water from a column (900 mm × 25 mm) of Bio-Gel P2. It was chromatographed on a column (200 mm × 5 mm) of Dowex-1 acetate with a linear gradient (0–0.1 M in 200 ml) of acetic acid. The product (51 mCi) was dried under reduced pressure and was dissolved in 10 mM-potassium phosphate buffer, pH 7.0, containing 10% (w/v) ethanol. High-pressure liquid chromatography showed that 90% of the radioactivity was present in 7-aminodesacetoxycephalosporanic acid. However, the product contained about 50% of an unlabelled contaminant that was probably 7-aminocephalosporanic acid. The

specific activity of the 7-aminodesacetoxycephalosporanic acid was about 0.5 Ci/mmol.

The 7-amino[3-*methyl*- ^3H]desacetoxycephalosporanic acid solution was evaporated to dryness under reduced pressure immediately before being used to prepare various 7-aminodesacetoxycephalosporanic acid derivatives. The unlabelled contaminant was assumed to be responsible for the impurity present in all the radioactive cephalosporins made from this 7-amino[3-*methyl*- ^3H]desacetoxycephalosporanic acid.

7 β -Formamido[3-methyl- ^3H]desacetoxycephalosporanic acid

Acetic anhydride (0.75 ml) was added to 98% (w/v) formic acid (4.0 ml) cooled in ice and a sample (40 μl) added to ice-cold 7-amino[3-*methyl*- ^3H]desacetoxycephalosporanic acid (5.1 mCi). After 15 min in ice the reaction was allowed to continue for a further 2 h at room temperature. The volatile components were removed under high vacuum and the residue was extracted with ethyl acetate (0.5 ml). The 7 β -formamidodesacetoxycephalosporanic acid was isolated by evaporation of the ethyl acetate extract and stored in 10 mM-potassium phosphate, pH 7.0, containing 10% (w/v) ethanol. The product (1.95 mCi) showed a radiochemical purity of better than 95% by high-pressure liquid chromatography.

7 β -Glutamido[3-methyl- ^3H]desacetoxycephalosporanic acid

Trimethylamine (5 μl) and glutaric anhydride (5.1 mg) in pyridine (22.5 μl) were added to ice-cold 7-amino[3-*methyl*- ^3H]desacetoxycephalosporanic acid (15 mCi) and the mixture stirred, first for 90 min cooled in ice, and then for 90 min at room temperature. The mixture was evaporated to dryness under vacuum, re-dissolved in 1 M-NaHCO₃ (1.0 ml), washed with ethyl acetate (3 $\times$ 0.4 ml), acidified with concentrated HCl (0.1 ml) and extracted with ethyl acetate (3 $\times$ 1.0 ml). The 7 β -glutamidodesacetoxycephalosporanic acid was evaporated to dryness under vacuum and the residue dissolved in 10 mM-potassium phosphate, pH 7.0, containing 10% (w/v) ethanol. The product (9.7 mCi) showed a radiochemical purity of better than 95% by high-pressure liquid chromatography.

7 β -Phenoxyacetamido[3-methyl- ^3H]desacetoxycephalosporanic acid

Phenoxyacetyl chloride (1.3 μl) in dry dichloromethane (0.1 ml) and 0.38 M-NaHCO₃ (0.5 ml) were added to ice-cold 7-amino[3-*methyl*- ^3H]desacetoxycephalosporanic acid (5.1 mCi) and the resulting two-phase mixture was stirred vigorously, first for 30 min

cooled in ice, and then for 30 min at room temperature. The dichloromethane was removed under vacuum and the residue acidified with dilute HCl. The 7 β -phenoxyacetamidodesacetoxycephalosporanic acid was extracted with ethyl acetate (1 ml), washed with water (1 ml), evaporated to dryness under vacuum and re-dissolved in 10 mM-potassium phosphate, pH 7.0, containing 10% (w/v) ethanol. When chromatographed on thin layers of silica (Merck 5735) in chloroform/methanol/acetic acid (18:2:1, by vol.) the 7-phenoxyacetamido[3-*methyl*- ^3H]desacetoxycephalosporanic acid (1.66 mCi) contained 95% of the radioactivity.

7 β -[2-(2-Furyl)-2-methoxyaminoacetamido][3-methyl- ^3H]desacetoxycephalosporanic acid (syn isomer)

A solution of *syn*-methoxyimino 2-furyl acetic acid (170 mg) in dry dichloromethane (20 ml) containing dry *NN*-dimethylformamide (1 drop) was cooled in ice and treated with triethylamine (0.14 ml) followed by oxalyl chloride (0.1 ml). The solution was stirred for 60 min while cooled in ice. A sample of the prepared solution (0.2 ml) was added to ice-cold 7-amino[3-*methyl*- ^3H]desacetoxycephalosporanic acid (5.1 mCi) in acetone (0.2 ml) and aq. 0.38 M-NaHCO₃ (1 ml). The resulting two-phase system was stirred vigorously for 2 h while cooled in ice. The mixture was evaporated under vacuum to remove organic solvents, acidified with dilute HCl and extracted into ethyl acetate. The extract was washed with water, evaporated to dryness and the product re-dissolved in 10 mM-potassium phosphate, pH 7.0, containing 10% (w/v) ethanol.

The product was chromatographed on a column (200 mm $\times$ 15 mm) of DEAE-cellulose DE-23, equilibrated with 10 mM-imidazole adjusted to pH 7.0 with HCl, in a linear gradient (0–0.2 M in 200 ml) of NaCl. The fractions that contained the 7 β -[2-(2-furyl)-2-methoxyiminoacetamido][3-*methyl*- ^3H]desacetoxycephalosporanic acid (1.86 mCi) were eluted at about 100 mM-NaCl. The bulked fractions (35 ml) were diluted to 50 ml, mixed with 5 ml of ethanol, and stored at 4°C. The product showed a radiochemical purity of better than 95% by high-pressure liquid chromatography.

7 β -([1- ^{14}C]Phenylacetamido)desacetoxycephalosporanic acid

The diphenyl methyl ester of 7-aminodesacetoxycephalosporanic acid (0.1 mmol) was dissolved in dry dichloromethane (2 ml) containing dicyclohexylcarbodi-imide (0.1 mmol). The solution was stirred at 20°C while a solution of [1- ^{14}C]phenylacetic acid (5 mCi at a sp. radioactivity of 59 mCi/mmol, obtained from The Radiochemical Centre, Amersham, Bucks.,

U.K.) in dichloromethane (5 ml) was added. After 4 h the precipitate was filtered off and washed with dichloromethane. The filtrate and washings were combined and dried to a solid that was dissolved in trifluoroacetic acid (0.12 ml) and anisole (3 drops). After 4 min the solvents were removed by an azeotropic distillation with toluene (3 × 5 ml).

The impure 7 β -([1-¹⁴C]phenylacetamido)desacetoxycephalosporanic acid was partitioned between dichloromethane (20 ml) and saturated NaHCO₃ (10 ml). The organic phase was washed with NaHCO₃ and the combined aqueous phases were washed with dichloromethane (3 × 10 ml). The aqueous phase was then acidified to pH 1.5 with 11.5 M-HCl and extracted with ethyl acetate (100 ml). The aqueous phase was washed with ethyl acetate (2 × 20 ml) and the combined organic phases were washed with water and saturated NaCl, and were then dried with MgSO₄. The ethyl acetate was evaporated off and the solid was chromatographed on DEAE-cellulose DE-23 in the acetate form.

The solid was dissolved in water (10 ml) and adjusted to pH 7 with 1 M-NH₃. This solution was loaded on a column (220 mm × 9 mm) of DEAE-cellulose DE-23 acetate equilibrated with water. The 7 β -([1-¹⁴C]phenylacetamido)desacetylcephalosporanic acid was eluted with a linear gradient (0–1 M in 500 ml) of acetic acid. The cephalosporin, which was eluted at a concentration of about 0.65 M-acetic acid, was dried and re-dissolved in 10 mM-potassium phosphate buffer, pH 6.8. The solution was adjusted to pH 6.0 with 1 M-acetic acid and stored at –10°C.

The product (3 mCi at a sp. radioactivity of about 55 mCi/mmol) had a radiochemical and chemical purity better than 98% by high-pressure liquid chromatography.

Unlabelled cephalosporin standards

Unlabelled cephalosporin and desacetoxycephalosporin standards were made by using similar techniques starting from 7-aminocephalosporanic acid and 7-aminodesacetoxycephalosporanic acid. Unlabelled desacetylcephalosporin standards were made by treating the cephalosporin analogues with an acetyl esterase (U.S. Patent, 1975).

Supplemented phosphate buffer

The supplemented phosphate buffer contained a mixture of 10 mM-KH₂PO₄ and 10 mM-K₂HPO₄ at pH 7.0, 0.1 mM-dithiothreitol and 10 μ M-desacetoxycephalosporin C.

Methods

Enzyme preparation

Cultures of *S. clavuligerus* (1.5 litres) and *A. chrysogenum* (0.5 litre) were grown and harvested as

described by Brewer *et al.* (1977a,b). The cells (90 g and 38 g wet wt. respectively) were suspended in the supplemented phosphate buffer (final volume about 180 ml) and were broken at 4°C in a Dynamill (Glen Creston, Stanmore, Middx., U.K.) with 250 ml of 0.1 mm glass beads stirred at 2000 rev./min for 2 min in the 300 ml glass head. The homogenates (160 ml) were centrifuged at 4°C (30000g for 30 min) and portions (120 ml) of the supernatants were chromatographed at 4°C on a column (50 mm × 200 mm) of DEAE-cellulose DE-32 equilibrated with the supplemented phosphate buffer. Protein was eluted from the column at 70 ml/h with a linear gradient (0–0.5 M in 1 litre) of NaCl in the supplemented phosphate buffer. Fractions (11 ml) were collected and assayed for desacetoxycephalosporin C dioxygenase activity. Peak fractions were pooled (about 110 ml) and concentrated at 4°C to 10 ml by ultrafiltration through a PM-10 membrane at 0.2 MPa (30 lb/in²). The concentrate was dialysed overnight at 4°C against the supplemented phosphate buffer. The dialysed enzyme was stored at –10°C.

Dioxygenase assay

Desacetoxycephalosporin C dioxygenase was assayed at 28°C in 0.1 ml of water that contained Fe(NH₄)₂(SO₄)₂ (10 nmol), 2-oxoglutarate (100 nmol), sodium ascorbate (100 nmol), dithiothreitol (100 nmol), Pipes buffer (500 nmol) adjusted to pH 6.75 with KOH, and [3-methyl-³H]desacetoxycephalosporin C (10 nmol at a sp. radioactivity of about 10 Ci/mol).

Dioxygenase purified from *A. chrysogenum* only displays full activity when preincubated with an incomplete reaction mixture. The activity of this enzyme was therefore measured after it had been preincubated for 10 min at 28°C in a reaction mixture from which the [3-methyl-³H]desacetoxycephalosporin C had been omitted. The oxygenation was then started by addition of this labelled substrate. The small amount of unlabelled desacetoxycephalosporin C carried over in the supplemented phosphate buffer (usually about 10 pmol) does not prevent the effect of this preincubation.

The dioxygenase from *S. clavuligerus* requires no such preincubation.

After a suitable time (usually 10–15 min) the dioxygenase reaction was stopped by adding acid. After treating the reaction mixtures with 10 μ l of acetic acid the product can be determined directly by high-pressure liquid chromatography. Alternatively, after treating them with 0.1 ml of 0.5 M-HCl for 30 min at 28°C the lactonized product can be eluted from Dowex-1 acetate (Brewer *et al.*, 1977b). Directly measured activities are somewhat greater than those measured after lactonization (Brewer *et al.*, 1977b), even though the lactonization itself is effectively complete.

Protein determination

Protein was determined by the A_{280} .

Measurement of radioactivity

All samples were diluted to 1 ml with water and were mixed with 15 ml of NE250, a dioxan-based scintillant. The radioactivity present in the sample was then measured in a Tricarb 3320 (Packard Instruments, Caversham, Berks., U.K.).

High-pressure liquid chromatography

The 7-aminocephalosporins were eluted from Partisil 10 SCX (Whatman) at about 22°C by using 1.2 g of $(\text{NH}_4)_2\text{HPO}_4$ in 1 litre of 40% (v/v) methanol/water adjusted to pH 4.4 with H_3PO_4 . The columns (250 mm × 4 mm) were eluted at a pressure of 5.5 MPa (800 lb/in²). The flow rate was about 1 ml/min. Under these conditions, for example, 7-amino-desacetylcephalosporanic acid and 7-aminodesacetoxycephalosporanic acid were eluted after 3.0 and 3.5 min respectively.

The 7 β -acylamidocephalosporins were eluted from ODS-modified 5 μ silica (Spherisorb; Phase Separations, Queensferry, Clwyd, U.K.) at 50°C with 33% (v/v) methanol/water adjusted to pH 2.2 with H_3PO_4 . The columns (200 mm × 4.6 mm) were eluted at a pressure of 3.5 MPa (500 lb/in²). The flow rate was about 1 ml/min. Under these conditions, for example, desacetylcephalosporin C, cephalosporin C and desacetoxycephalosporin C were eluted after 3.6, 4.7 and 5.1 min respectively. The methanol was omitted from the eluent during chromatography of 7 β -formamidodesacetoxycephalosporanic acid.

The measured elution times are only a guide and will vary from one batch of column packing to another.

The u.v.-absorbing material eluted from the columns was detected continuously at 260 nm with

a CE212 (Cecil Instruments, Cambridge, U.K.) with an 8 μ l flow cell. Any radioactive label that was present was measured continuously after mixing the effluent from the CE212 with scintillant (10 vol.). This scintillant contained 6 g of 5-(biphenyl-4-yl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole dissolved in 1 litre of toluene and mixed with 500 ml of Triton X-100. The radioactivity was then detected in an LSCI (Nuclear Enterprises) fitted with a Teflon tube (1.14 mm internal diam.) as a flow cell (nominal volume 2 ml). The overall design of the apparatus was similar to that described elsewhere (Reeve & Crozier, 1977).

Results

Purification

Desacetoxycephalosporin C dioxygenase was isolated from both *S. clavuligerus* and *A. chrysogenum* (Table 1). DEAE-cellulose is not easily equilibrated with the buffer containing the 5 mM-2-oxoglutarate and 5 mM-ascorbate that was originally used to stabilize the enzyme while the cells were broken (Brewer *et al.*, 1977b). Moreover, the ascorbate, which absorbs strongly at 265 nm, swamps the absorption at 280 nm caused by the protein eluted from the DEAE-cellulose. Cells were therefore broken in buffer containing 0.1 mM-dithiothreitol and 10 μ M-desacetoxycephalosporin C. Under these conditions both the enzymes are eluted from DEAE-cellulose DE-32 with about 0.2 M-NaCl, somewhat after the main protein peaks, and they are purified 10–15-fold. The ultra-filtration step results in a further increase in specific activity, yielding products from *S. clavuligerus* and *A. chrysogenum* that are about 20- and 30-fold purified over the original supernatants respectively.

Both enzymes are stable for at least 48 h in 1 M-NaCl at 4°C, although the rate at which they oxidize desacetoxycephalosporin C is decreased if the NaCl

Table 1. Purification of desacetoxycephalosporin C dioxygenase from *S. clavuligerus* and *A. chrysogenum*. The fractions were prepared and then assayed by the Dowex-1 acetate procedure described in the Methods section.

Fraction	<i>A. chrysogenum</i>					<i>S. clavuligerus</i>				
	Dioxygenase activity					Dioxygenase activity				
	Vol. (ml)	Protein concn. (A_{280})	Units/litre	Specific activity (units/ A_{280})	% recovery	Vol. (ml)	Protein concn. (A_{280})	Units/litre	Specific activity (units/ A_{280})	% recovery
Broken-cell supernatant	160	41.1	28.0	0.00067	100	580	16	3.69	0.00023	100
DEAE-cellulose eluate	150	3.55	25.2	0.0071	84.5	100	6.2	20.2	0.0033	94
PM-10 concentrate	7.0	—	—	—	—	11.5	31.2	—	—	—
Dialysate	8.6	16.5	355	0.021	64.6	14.4	27.9	134	0.0048	90

Fig. 1. Effect of NaCl on the dioxxygenase from *A. chrysogenum*.

A sample (50 μ l) of the enzyme preparation from *A. chrysogenum* was preincubated for 15 min at 28°C with 50 μ l of water that contained Pipes (0.5 μ mol) adjusted to pH 6.75 with KOH, $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ (10 nmol), 2-oxoglutarate (100 nmol), sodium ascorbate (100 nmol) and dithiothreitol (100 nmol). The preincubation mixture was then diluted to 1 ml and 20 μ l was transferred to the full incubation mixture and assayed by the Dowex-1 acetate procedure as described in the Methods section. The incubation mixture also contained NaCl as indicated.

Table 2. Effect of preincubation on the activity of the dioxxygenases from *S. clavuligerus* and *A. chrysogenum*.

The dioxxygenases were purified and then assayed by the Dowex-1 acetate procedure described in the Methods section. The enzyme preparations were assayed with and without the preincubation described in the text for the *A. chrysogenum* dioxxygenase.

Dioxxygenase		Desacetyl- cephalosporin C formed (nmol/min per ml of enzyme)	
Source of enzyme	Volume in incubation (μ l)	With pre- incubation	Without pre- incubation
<i>S. clavuligerus</i>	2	152	154
<i>A. chrysogenum</i>	0.8	416	25

is not removed (Fig. 1). After dialysis against the supplemented phosphate buffer they have half-lives of about 1 month at -10°C.

The enzymes are less stable when they are purified on DEAE-cellulose DE-23 instead of DE-32. The activity is eluted at about 0.4M-NaCl, and quickly

Table 3. Substrate and cofactor requirements of the dioxxygenase from *S. clavuligerus*.

The dioxxygenase was purified and then assayed by the Dowex-1 acetate procedure described in the Methods section. Each reaction mixture contained 1.3 μ l of enzyme preparation. Reaction components were omitted as indicated.

Omissions	Dioxxygenase activity (units/litre of enzyme)
None	128
Fe^{2+}	70
Dithiothreitol + ascorbate	12
2-Oxoglutarate	5
Fe^{2+} , dithiothreitol + ascorbate	2
Fe^{2+} , 2-oxoglutarate	3
Dithiothreitol + ascorbate, 2-oxoglutarate	6
Fe^{2+} , dithiothreitol + ascorbate, 2-oxoglutarate	1

disappears at 4°C, even after dialysis. All the results now reported were obtained with dioxxygenase purified on DE-32.

Preincubation

The activity of the dioxxygenase from *A. chrysogenum* increases when it is preincubated with some of its substrates and cofactors (Table 2). In contrast, preincubation does not increase the activity of the dioxxygenase from *S. clavuligerus*.

Cofactors

A reducing agent, Fe^{2+} cations and 2-oxoglutarate are all required before homogenates of *A. chrysogenum* will oxidize the 3-methyl group of desacetoxycephalosporin C (Brewer *et al.*, 1977b). The need to preincubate the purified enzyme before the full dioxxygenase activity is observed makes it difficult to decide which of these factors are required for the activation of the dioxxygenase and which are required for the oxidation of the 3-methyl group. In contrast, the cofactors required by the enzyme from *S. clavuligerus*, which does not require preincubation, are more easily discerned (Table 3).

This dioxxygenase is fully active only in the presence of 2-oxoglutarate, Fe^{2+} cations and reducing agents (ascorbate and dithiothreitol). The enzyme is inactive if either the 2-oxoglutarate or the reducing agents are omitted. The small amount of dithiothreitol (0.1 nmol) added with the purified enzyme is obviously insufficient to catalyse any oxidation of the 3-methyl group. If Fe^{2+} cations are omitted about half the full activity is retained (Table 3). Full activity also requires both ascorbate and dithiothreitol (Table 4), though each are 70 or 80% as effective as the combination. Glutathione was the only other reducing agent found to catalyse the oxidation, but it was much less effective than ascorbate or dithiothreitol.

Table 4. Effect of reducing agents on the activity of the dioxygenase from *S. clavuligerus*

The dioxygenase was purified and then assayed by the Dowex-1 acetate procedure described in the Methods section. Ascorbate and dithiothreitol were omitted from some reaction mixtures and were replaced with other reducing agents as indicated. The final concentration of each reducing agent was 10mM. Each reaction mixture contained 1.5 μ l of enzyme preparation.

Reducing agent added	Dioxygenase activity (units/litre of enzyme)
None	9
Dithiothreitol	77
Ascorbate	69
Dithiothreitol+ascorbate	96
Sodium dithionite	1
Sodium metabisulphite	3
NADH	7
2-Mercaptoethanol	7
Glutathione (reduced)	24

Table 5. Effect of nucleotides on the dioxygenase from *S. clavuligerus*

The dioxygenase was purified and then assayed by the Dowex-1 acetate procedure described in the Methods section. Ascorbate and dithiothreitol were omitted from some reaction mixtures. Nucleotides were added as indicated to give the final concentrations shown. Each reaction mixture contained 1.5 μ l of enzyme.

Nucleotide added	Concn. (mM)	Dioxygenase activity (units/litre of enzyme)	
		With reducing agent	Without reducing agent
None		103	26
ATP	10	8	8
	1	93	26
ADP	10	3	1
	1	96	14
GTP	10	5	1
	1	85	20

Effect of nucleotides

High concentrations of purine nucleotides inhibit the dioxygenase from *S. clavuligerus*, whereas lower concentrations have little if any effect (Table 5). They also have no effect on the enzyme's requirement for a reducing agent.

pH optimum

The dioxygenases from both *S. clavuligerus* and *A. chrysogenum* have similar pH optima centred at about pH 7 (Fig. 2).

Fig. 2. Effect of pH on the dioxygenases from *A. chrysogenum* and *S. clavuligerus*

A sample (25 μ l) of the enzyme preparation from *A. chrysogenum* was preincubated for 15 min at 28°C with 50 μ l of water that contained Pipes (5 μ mol) adjusted to pH 6.75 with KOH, Fe(NH₄)₂(SO₄)₂ (10 nmol), 2-oxoglutarate (1 μ mol), sodium ascorbate (1 μ mol) and dithiothreitol (1 μ mol). The preincubation mixture was then diluted to 1.5 ml with water. Samples (40 μ l) of this diluted preincubation mixture or of an *S. clavuligerus* enzyme preparation (4 μ l) were then transferred to a full incubation mixture similar to that described in the Methods section, except that the Pipes buffer was replaced by various other buffers (5 μ mol) each adjusted to the required pH with KOH. The product was assayed by the Dowex-1 acetate procedure (see Methods section). The buffers used were acetic acid (●), 4-morpholine-ethanesulphonic acid (□, ■), Pipes (△, ▲), 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (▽, ▼) and 2-amino-2-methyl propane-1,3-diol (◇, ◆). Open symbols denote assays with the dioxygenase from *S. clavuligerus*, and closed symbols those with the dioxygenase from *A. chrysogenum*.

Cephalosporin specificity

Both dioxygenases are highly specific for des-acetoxycephalosporin C. When the amino adipamide side chain is removed, or replaced by another acid amide (Fig. 3), the 3-methyl group is not oxidized at a detectable rate. Rates of oxidation that were >1% of those for the oxidation of desacetoxycephalosporin C would have been detected.

Preincubation of dioxygenase from *A. chrysogenum*

The need to preincubate the *A. chrysogenum* dioxygenase before it will show its full activity clearly distinguishes it from the *S. clavuligerus* enzyme. If the *A. chrysogenum* enzyme is incubated with a complete reaction mixture (desacetoxycephalosporin C, 2-oxoglutarate, Fe²⁺ cations and reducing agents) its full activity is only slowly recovered (Fig. 4). However, if 2-oxoglutarate is briefly omitted the 3-methyl group is oxidized as soon as it is added (Fig. 5). This effect is similar to that observed when the other substrate, desacetoxycephalosporin C, is the reaction

Formula	7 β -Side chain	Radioactive label
5-D-Amino adipamido (desacetoxycephalosporin C)	[3- <i>methyl</i> - ^3H]
Amino (7-aminodesacetoxycephalosporanic acid)	[3- <i>methyl</i> - ^3H]
Formamido	[3- <i>methyl</i> - ^3H]
Phenylacetamido	[1- ^{14}C]Phenylacetamido
Phenoxyacetamido	[3- <i>methyl</i> - ^3H]
Glutaramido	[3- <i>methyl</i> - ^3H]
2-(2-Furyl)-2-methoxyimino acetamido (<i>syn</i> isomer)	[3- <i>methyl</i> - ^3H]

Fig. 3. 3-Methylcephem derivatives tested as dioxxygenase substrates

The oxidation of the 3-methylcephem derivatives was tested as described in the Methods section. About 0.003 unit of dioxxygenase from *S. clavuligerus* or *A. chrysogenum* and about 0.4 nmol of [^3H]cephalosporin or about 5 nmol of [^{14}C]cephalosporin were used. Reaction mixtures that contained dioxxygenase from *A. chrysogenum* were preincubated at 28°C for 30 min before cephalosporin was added. At 30 min after addition of the cephalosporin the reactions were stopped with 10 μl of 1 M-acetic acid. Samples (10–20 μl) of the clarified reaction mixtures were then chromatographed at high pressure as described in the Methods section. Radioactivity in the peaks that corresponded to the 3-methyl and 3-hydroxymethyl derivatives was then measured.

component that is initially omitted. Under these conditions the enzyme is fully activated by a 10 min preincubation (Fig. 6).

When each of the reaction components is added individually only Fe^{2+} cations activate the dioxxygenase (Table 6), but they only reveal about 25% of

Fig. 4. Effect of incubation time on the dioxygenase activity of an enzyme preparation from *A. chrysogenum*

Samples (10 μ l) of dioxygenase prepared from *A. chrysogenum* as described in the Methods section were incubated at 28°C in 0.1 ml of water that contained Pipes buffer, pH 6.75, $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$, 2-oxoglutarate, sodium ascorbate, dithiothreitol and [3-methyl- ^3H]desacetoxycephalosporin C as described in the Methods section. The dioxygenase was not preincubated. The amount of product formed at various times was estimated by use of the Dowex-1 acetate procedure (see Methods section). The specific activity of the fully active enzyme preparation was 0.0033 unit/ A_{280} .

the enzyme's activity under the standard activation conditions (preincubation with Fe^{2+} cations, dithiothreitol and ascorbate, and 2-oxoglutarate). In contrast, when the dioxygenase is preincubated with Fe^{2+} cations and one of the substrates, either desacetoxycephalosporin C or 2-oxoglutarate, no activation occurs (Table 7). The small amount (about 10 pmol) of desacetoxycephalosporin C carried over from the enzyme preparation itself is obviously too little to prevent the activation by Fe^{2+} cations. Alone, neither of the two reducing agents (dithiothreitol and ascorbate) substantially affects the activation by Fe^{2+} ions, but together they do increase the activity, perhaps as much as 2-fold (Table 7).

Although the combined effect of dithiothreitol and Fe^{2+} cation is only slightly greater than the effect of Fe^{2+} cation alone, the two together do activate the enzyme in the presence of either desacetoxycephalosporin C or 2-oxoglutarate (Table 8). This action is quite different from the effect of the substrates on the activation caused by Fe^{2+} cation in the absence of dithiothreitol (Table 7). The reducing agent obviously protects that small amount of activation caused by

Fig. 5. Effect of incubation without 2-oxoglutarate on the activity of the dioxygenase from *A. chrysogenum*

Samples (5 μ l) of dioxygenase prepared from *A. chrysogenum* as described in the Methods section were incubated at 28°C in 0.1 ml of water that contained Pipes buffer, pH 6.75, $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$, sodium ascorbate, dithiothreitol and [3-methyl- ^3H]desacetoxycephalosporin C as described in the Methods section. The dioxygenase was not preincubated. 2-Oxoglutarate (100 nmol) was added to half the reaction mixtures (●) at the start of the incubation, and to the other half (○) after 5 min at 28°C. The amount of product formed at various times was estimated by use of the Dowex-1 acetate procedure. The specific activity of the fully active enzyme was 0.0018 unit/ A_{280} .

the Fe^{2+} cations alone. When both reducing agents (ascorbate and dithiothreitol) are present with the Fe^{2+} cations either desacetoxycephalosporin C (Fig. 5) or 2-oxoglutarate (Fig. 6, Tables 2, 6 and 7) will activate the enzyme.

The full activity observed is usually more than 10 times that observed with preparations that are not preincubated (Table 2).

Discussion

The ability of the cell-free extracts from *A. chrysogenum* and *S. clavuligerus* to oxidize [3-methyl- ^3H]desacetoxycephalosporin C to [3-hydroxymethyl- ^3H]desacetylcephalosporin C confirms that the oxidation of the 3-methyl group on the cephem nucleus is a late stage in cephalosporin biosynthesis. It also points to a common pathway for cephalosporin biosynthesis in actinomycetes and fungi. The enzymes responsible, which appear when *A. chrysogenum* and *S. clavuligerus* are actively synthesizing cephalosporins, are not mono-oxygenases (Liersch *et al.*, 1976; Fujisawa *et al.*, 1977). They are intermolecular dioxygenases (see Hayashi, 1974) that use 2-oxoglutarate and molecular O_2 as co-substrates.

Fig. 6. Effect of preincubation time on the activity of the dioxxygenase from *A. chrysogenum*

Samples (0.8 μ l) of dioxxygenase from *A. chrysogenum* were preincubated at 28°C in 0.1 ml of water that contained Pipes buffer, pH 6.75, $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$, 2-oxoglutarate, sodium ascorbate and dithiothreitol as described in the Methods section. After various times [3-*methyl*- ^3H]desacetoxycephalosporin C (10 nmol) was added and in each case the incubation was continued for a further 10 min. The amount of product formed was estimated by the Dowex-1 acetate procedure.

Table 6. Preincubation of dioxxygenase from *A. chrysogenum* with various components of the reaction mixture

The dioxxygenase from *A. chrysogenum* was purified and then assayed by the Dowex-1 acetate procedure described in the Methods section, except that the preincubation conditions were changed. Enzyme preparation (0.8 μ l) was incubated for 15 min at 28°C with 0.5 μ mol of Pipes buffer, pH 6.75, and with those components of the reaction mixture indicated. At the end of the preincubation all the other components of the reaction mixture were added to give a final volume of 0.1 ml. The reaction was stopped after 10 min with 0.1 ml of 1 M-HCl.

Additions to preincubation (nmol added)	Pre-incubation volume (μ l)	Dioxxygenase activity (units/litre of enzyme)
None	50	30
Fe^{2+} (10)	60	119
[3- <i>methyl</i> - ^3H]Desacetoxycephalosporin C (10)	60	17
2-Oxoglutarate (100)	60	21
Dithiothreitol (100)	60	30
Ascorbate (100)	60	25
Fe^{2+} (10), dithiothreitol (100), ascorbate (100), 2-oxoglutarate (100)	90	416

Table 7. Preincubation of dioxxygenase from *A. chrysogenum* with Fe^{2+} cations and various components of the reaction mixture

The dioxxygenase from *A. chrysogenum* was purified and then assayed by the Dowex-1 acetate procedure described in the Methods section, except that the preincubation conditions were changed as described in Table 6. Enzyme preparation (0.8 μ l) was used in each reaction mixture.

Additions to preincubation (nmol added)	Pre-incubation volume (μ l)	Dioxxygenase activity (units/litre of enzyme)
Fe^{2+} (10)	60	101
Fe^{2+} (10), [3- <i>methyl</i> - ^3H]desacetoxycephalosporin C (10)	70	42
Fe^{2+} (10), 2-oxoglutarate (100)	70	26
Fe^{2+} (10), dithiothreitol (100)	70	132
Fe^{2+} (10), ascorbate (100)	70	105
Fe^{2+} (10), ascorbate (100), dithiothreitol (100)	80	172
Fe^{2+} (10), dithiothreitol (100), ascorbate (100), 2-oxoglutarate (100)	90	387

Table 8. Preincubation of the dioxxygenase from *A. chrysogenum* with Fe^{2+} cations and reducing agents

The dioxxygenase from *A. chrysogenum* was purified and then assayed by the Dowex-1 acetate procedure described in the Methods section, except that the preincubation conditions were altered as described in Table 6. Enzyme preparation (0.8 μ l) was used in each reaction mixture.

Additions to preincubation (nmol added)	Pre-incubation volume (μ l)	Dioxxygenase activity (units/litre of enzyme)
None	50	26
Fe^{2+} (10), dithiothreitol (100)	70	119
Fe^{2+} (10), dithiothreitol (100), 2-oxoglutarate (100)	80	132
Fe^{2+} (10), dithiothreitol (100), [3- <i>methyl</i> - ^3H]desacetoxycephalosporin C (10)	80	100

The handful of other known 2-oxoglutarate dioxxygenases all have common properties. They all use 1 atom from molecular O_2 to decarboxylate 2-oxoglutarate, while incorporating the other into a

hydrophilic co-substrate. They all require Fe^{2+} cations and a reducing agent, usually ascorbate, and they are all specific for 2-oxoglutarate and for their own particular co-substrate (Abbott & Udenfriend, 1974). The desacetoxycephalosporin C dioxygenases from *A. chrysogenum* and *S. clavuligerus* obviously belong to this group of enzymes and seem to show the same degree of specificity for their particular co-substrate. Certainly this seems likely from experiments with a small but varied group of substrates (Fig. 3), but it is not yet clear whether their specificity is restricted to a 3-methyl cephem carrying a 7β -D-aminoadipyl side chain. Of the two the enzyme from *S. clavuligerus* may have the broader specificity. Occasionally when D-norvaline was present, it seemed to oxygenate a 7β -formamidodesacetoxycephalosporanic acid, but this reaction was barely detectable even when it was observed. Also *S. clavuligerus* itself may oxygenate a 7α -methoxy-3-methylcephem nucleus during the biosynthesis of the cephamycins (Whitney *et al.*, 1972), but the oxygenation could equally well precede the methylation.

The degree of specificity that the desacetoxycephalosporin C dioxygenases show for 2-oxoglutarate is not known; a few preliminary experiments with 2-oxoadipate and oxaloacetate suggest that neither can replace 2-oxoglutarate, but this requires confirmation. However, the enzymes are not absolutely dependent on ascorbate; dithiothreitol is equally effective and even glutathione will allow some reaction to take place. This requirement of the dioxygenase for a reducing agent is best supplied by a mixture of ascorbate and dithiothreitol.

Thymine 7-hydroxylase (Holme, 1975), which also belongs to this group of 2-oxoglutarate-linked dioxygenases, catalyses a reaction that is very similar to that catalysed by the desacetoxycephalosporin C dioxygenases. However, this enzyme not only oxidizes thymine (5-methyluracil) to 5-hydroxymethyluracil, but also continues the oxidation to uracil-5-carbaldehyde and uracil-5-carboxylate. Whether the desacetoxycephalosporin C dioxygenase will carry out a similar sequence of reactions remains an open question. The assays that detect either [3-hydroxymethyl- ^3H]desacetylcephalosporin C after high-pressure liquid chromatography or its lactone after chromatography on Dowex-1 acetate show that little if any ^3H is lost as $^3\text{H}_2\text{O}$ during the oxidation of the 3-methylcephem to a 3-hydroxymethylcephem (Brewer *et al.*, 1977b). Yet no more than 80% of the radioactivity in the [3-methyl- ^3H]desacetoxycephalosporin C is ever recovered as [3-hydroxymethyl- ^3H]desacetylcephalosporin C. However, the further oxidation to a cephem-3-carbaldehyde would be only one of a number of explanations for this discrepancy. For example, if the D-aminoadipamido side chain of the desacetylcephalosporin was further attacked by a D-amino acid oxidase, no product

would be detected by either assay. On the other hand, a cephem-3-carbaldehyde is a likely product of cephalosporin fermentations (Fujisawa & Kanzaki, 1975), and the desacetoxycephalosporin C dioxygenase could well catalyse its production.

The need to preincubate the dioxygenase from *A. chrysogenum* clearly distinguishes it from the enzyme from *S. clavuligerus*, but forces a comparison with proline hydroxylase, another 2-oxoglutarate-linked dioxygenase that is activated by preincubation with Fe^{2+} cations, 2-oxoglutarate and ascorbate (Cardinale *et al.*, 1975). However, nucleotides do not activate the desacetoxycephalosporin C dioxygenases as they do proline hydroxylase (Takeda *et al.*, 1976).

The effect of the aerobic preincubation on the activity of the desacetoxycephalosporin C dioxygenase is dramatic, but why the activity should be so affected is not clear. An important role is obviously played by Fe^{2+} cations that can activate the enzyme by themselves, albeit in the presence of O_2 . O_2 may or may not be crucial, but unless Fe^{2+} cations are added (Tables 6 and 7) the enzyme is certainly inactive when preincubated aerobically with any of the other components. In contrast the enzyme from *S. clavuligerus* is relatively independent of added Fe^{2+} cations (Table 3).

The enzymes from *A. chrysogenum* and *S. clavuligerus* may differ in their ability to bind Fe^{2+} cations. The aerobic preincubation with Fe^{2+} cations, reducing agents, and either 2-oxoglutarate or desacetoxycephalosporin C could then be necessary to present the iron to the dioxygenase in an optimal state. Ascorbate, which has both reducing and chelating properties (Lewin, 1976), may have an important role in the transfer of these Fe^{2+} cations, but it can be replaced by dithiothreitol. The transfer is quite slow and the enzyme usually needs about 10 min to reach full activity, but why the transfer should be inhibited when 2-oxoglutarate or desacetoxycephalosporin C are added without a reducing agent (Table 7) is not clear. Of the enzyme's three substrates O_2 , by oxidizing the Fe^{2+} cations, is the one that might have been expected to have this effect.

The parallels between the desacetoxycephalosporin C dioxygenase, thymine 7-hydroxylase and proline hydroxylase are intriguing; indeed the common properties of all these 2-oxoglutarate-linked dioxygenases pose an interesting problem in comparative biochemistry. The enzymes from *A. chrysogenum* and *S. clavuligerus* also provide a clear enzymic link between the 3-methyl- and the 3-hydroxymethyl-cephem nuclei during cephalosporin biosynthesis. It now remains to identify the enzymes responsible for the two and three stages of dehydrogenation that link the tripeptide precursor with a 2,2-dimethylpenam and a 3-methylcephem nucleus. That alone will show whether penicillins are the direct precursors of cephalosporins or whether the

two nuclei are the product of separate ring-closure reactions (Kanzaki & Fujisawa, 1976).

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