DEACETOXYCEPHALOSPORIN C SYNTHETASE AND DEACETOXYCEPHALOSPORIN C HYDROXYLASE ARE TWO SEPARATE ENZYMES IN *STREPTOMYCES CLAVULIGERUS*

SUSAN E. JENSEN, DONALD W. S. WESTLAKE and SAUL WOLFE†

Department of Microbiology, University of Alberta, Edmonton, Alberta, Canada T6G 2E9
†Department of Chemistry, Queen's University, Kingston, Ontario, Canada K7L 3N6

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Streptomyces clavuligerus produces a number of β-lactam compounds including penicillin N, cephamycin C and clavulanic acid. The synthesis of the penicillin and cephamycin compounds is accomplished by means of a biosynthetic pathway which closely parallels the corresponding pathway to cephalosporin C in *Cephalosporium acremonium*. Two of the enzymes involved in this pathway, deacetoxycephalosporin C (DAOC) synthetase and DAOC hydroxylase, carry out sequential reactions in which penicillin N undergoes ring expansion to DAOC and then DAOC is hydroxylated at the C3 methyl group to give deacetylcephalosporin C (DAC). These two enzymes are intermolecular dioxygenases which require molecular oxygen, iron, ascorbate and α-ketoglutarate for activity in both *C. acremonium*[1~4] and *S. clavuligerus*[5~6].

In view of the similar cofactor requirements of DAOC synthetase and DAOC hydroxylase, SCHEIDEGGER *et al.*[7] recently undertook a purification and comparison of the two enzymes in cell-free extract from *C. acremonium*. They were unable to separate the two enzyme activities by any of a variety of protein purification techniques. On this basis, they have suggested that DAOC synthetase and DAOC hydroxylase activities may both reside in a single bifunctional enzyme in *C. acremonium*.

In previous studies with *S. clavuligerus*[8], it was found that DAOC hydroxylase activity was absent from DAOC synthetase purified by anion exchange chromatography. The separate existence of DAOC hydroxylase was not determined because of the lack of an assay procedure for this activity. Such a procedure has now been developed, and it has been found that the synthetase and hydroxylase activities reside in different enzymes.

Cell-free extract was prepared from *S. clavuligerus* and partially purified by streptomycin sulfate and ammonium sulfate precipitation as previously described[8]. Partially-purified enzyme from 1 liter of original culture was then applied to a DEAE-trisacryl column (1.6×30 cm) which had been equilibrated with TDE buffer (0.05 M Tris-HCl (pH 7.0) - 1.0 mM dithiothreitol - 0.01 mM EDTA). The column was washed with 50 ml of TDE buffer and eluted with a linear gradient of 200 ml each of normal TDE buffer and TDE buffer containing 0.2 M Tris-HCl. Fractions of 50 drops (2.5 ml) were collected and monitored for UV-absorption at 280 nm. DAOC synthetase and DAOC hydroxylase were measured in reaction mixtures containing 2.8 mM sodium ascorbate, 0.045 mM $FeSO_4$, 1 mM α-ketoglutarate, 7.5 mM KCl, 7.5 mM $MgSO_4$, 0.05 M Tris-HCl (pH 7.0) and 0.03 ml of enzyme in a final volume of 0.04 ml. Each reaction mixture also contained 4 μg of either penicillin N (DAOC synthetase) or DAOC (DAOC hydroxylase). Reactions were incubated at 20°C

Fig. 1. Location of DAOC hydroxylase activity by TLC followed by biological assay.

Two microlitre amounts of reaction mixtures were applied to TLC sheets, developed in 1-BuOH - AcOH - H_2O (3: 1: 1) and then bioassayed on agar slabs inoculated with *E. coli* Ess.

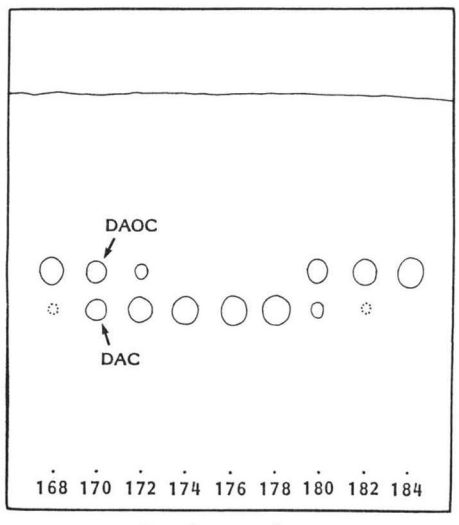


Fig. 2. Ion exchange chromatography of cell-free extract from *S. clavuligerus*.
Partially purified cell-free extract was applied to a DEAE-trisacryl column and eluted with a Tris-HCl gradient.

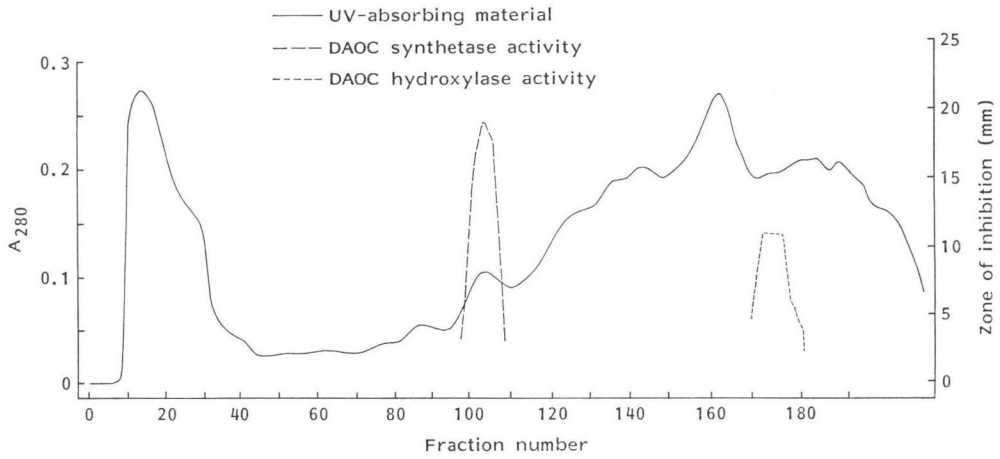
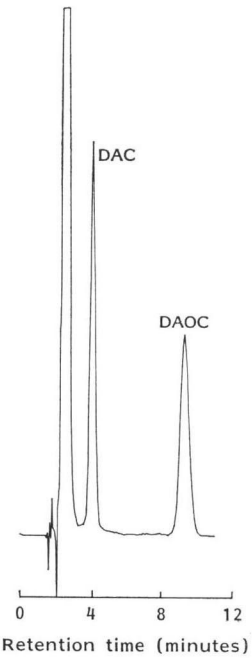


Fig. 3. Analysis of DAOC hydroxylase activity by HPLC.
Twenty microlitre amounts of reaction mixtures were analysed with a mobile phase of 5% MeOH 95% KH_2PO_4 (0.05 M, adjusted to pH 4.0 with concentrated H_3PO_4) supplemented with 0.002% tetrabutylammonium bromide.



for 1 hour and terminated by the addition of 0.04 ml of MeOH. DAOC synthetase activity was detected in reaction mixtures (20 μl) by agar disc diffusion on agar inoculated with *Escherichia coli* Ess as indicator organism and containing penicillinase at 1,000 units/ml as previously described[6].

The hydroxylase activity was detected by TLC of reaction mixtures and biological assay of the developed chromatograms. Reaction mixtures were centrifuged for 5 minutes at 12,000 $\times g$ and 2 μl amounts of supernatants were applied to cellulose TLC sheets. The chromatograms were developed 1-BuOH - AcOH - H_2O (3: 1: 1), dried thoroughly and then placed face down on agar slabs inoculated with *E. coli* Ess. After 30 minutes at 21°C the cellulose sheets were removed and the agar slabs were incubated overnight at 37°C. The hydroxylase activity was clearly detectable by the disappearance of the zones of inhibition due to DAOC and the appearance of zones of inhibition due to DAC (Fig. 1). Zones of inhibition due to DAC were measured, as an estimate of DAOC hydroxylase activity.

Anion exchange chromatography cleanly separated DAOC synthetase from DAOC hydroxylase (Fig. 2). No evidence of overlapping or cross reacting activities was found for either enzyme. The synthetase eluted early, just after the application of the gradient. The hydroxylase eluted much later. Using the same anion exchange resin (DEAE-trisacryl), SCHEIDEGGER *et al.*[7] were unable to obtain any separation between hydroxylase and synthetase activities from *C. acremonium*. A comparison of the elution profiles of these enzymes from the two different organisms indicates that the DAOC hydroxylases

have similar characteristics, but the DAOC synthetase of *S. clavuligerus* differs from that of *C. acremonium* in its weak binding to DEAE-trisacryl[4,7].

Both DAOC synthetase and DAOC hydroxylase activities from *S. clavuligerus* are stable after anion exchange chromatography, and are amenable to further investigation. When the partially purified enzymes were applied separately to a Sephadex G-200 superfine gel filtration column which had been calibrated with protein molecular weight markers, molecular weight estimates of 29,500 (DAOC synthetase) and 26,200 (DAOC hydroxylase) were obtained.

Quantitative measurement of DAOC synthetase activity by ion pair reverse phase HPLC has been described previously[8]. The same system (C_{18} column, stationary phase; 0.002% tetrabutylammonium bromide in 0.05 M KH_2PO_4 [pH 4.0] - MeOH (95: 5), mobile phase) was used to measure DAOC hydroxylase activity. Centrifuged reaction mixtures (20 μl) were analysed at a flow rate of 2 ml/minute with detection at 260 nm (Fig. 3). Complete baseline separation of DAOC (retention time 9.3 minutes) from DAC (retention time 4.0 minutes) was routinely achieved and interference by α-ketoglutarate (retention time 4.0 minutes) was eliminated by carrying out detection at 260 nm.

Two separable enzymes are thus responsible for DAOC synthetase and DAOC hydroxylase activities in *S. clavuligerus*. This situation differs markedly from the inseparable DAOC synthetase-hydroxylase of *C. acremonium*, and represents the first major difference which has been observed between the shared portions of the prokaryotic and eukaryotic biosynthetic pathways to cephalosporin.

Acknowledgements

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