PARTIAL PURIFICATION AND CATALYTIC PROPERTIES OF A BIFUNCTIONAL ENZYME IN THE BIOSYNTHETIC PATHWAY OF β-LACTAMS IN *CEPHALOSPORIUM ACREMONIUM*

A. SCHEIDEGGER, M. T. KÜENZI and J. NÜESCH

Research Laboratories of the Pharmaceutical Division of CIBA-GEIGY Limited, CH-4002 Basle, Switzerland

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The catalytic properties of the partially purified deacetoxycephalosporin C (DAOC)-synthetase and DAOC-hydroxylase from an industrial strain of *Cephalosporium acremonium* were studied. After mechanical breakage of the cells, purification was achieved by fractional $(NH_4)_2SO_4$ precipitation, gel chromatography on Sephadex G-75, ion exchange chromatography on DEAE-Trisacryl M and two isoelectric focusing steps. The two enzyme activities could not be separated. Indirect evidence was obtained from SDS-polyacrylamide gel electrophoresis of the purest fractions obtained by isoelectric focusing that the two reactions are catalyzed by a single enzyme with a molecular weight of 33,000±2,000 and a pI of 4.6±0.1. Both reactions require α-ketoglutarate, $FeSO_4$, ascorbate and O_2, whereas additional ATP shows only a slight stimulation.

According to present knowledge, cephalosporin C biosynthesis by *Cephalosporium acremonium* starts with the formation of the tripeptide[1] and proceeds *via* cyclization[2,3,4,5], epimerization[6,7,8] and ring expansion to deacetoxycephalosporin C (DAOC)[5,9,10,11]. DAOC is hydroxylated to give deacetylcephalosporin C (DAC)[12,13] from which cephalosporin C is formed by acetylation[14,15].

During the industrial production of cephalosporin C, the undesirable precursors penicillin N, DAOC and DAC are released into the culture medium in varying amounts, depending on the cultivation conditions and mutants used. Since strains giving high yields of cephalosporin C also form moderate quantities of penicillin N even under optimized conditions, the ring expansion reaction might be a limiting step in cephalosporin C biosynthesis. Recently, DAOC-synthetase has been partially purified and characterized by KUPKA *et al.*[5,9]. The enzyme was unstable and the activity was lost during further purification. The molecular weight was determined to be 31,000±3,000 and the *Km* for penicillin N 0.03 mM. Isopenicillin N was not accepted as a substrate. To carry out the reaction, α-ketoglutarate and $FeSO_4$ were required and the activity was stimulated by ascorbate and ATP. It was pointed out by KUPKA *et al.*[5] that the enzyme in the biosynthetic pathway, DAOC-hydroxylase, appears to require the same cofactors and reaction conditions. In *Streptomyces clavuligerus* both enzyme activities were demonstrated and also their requirement for α-ketoglutarate determined[12,13,16].

As a first step towards a more detailed understanding of the regulation of the terminal reactions in the biosynthetic pathway of cephalosporin C, a procedure was established to purify small amounts of DAOC-synthetase and hydroxylase from a high yielding mutant. Evidence was obtained that the ring expansion of penicillin N and the oxygenation of DAOC are catalyzed by a single dioxygenase.

Materials and Methods

Materials

α-Ketoglutarate and the protein test solution were obtained from Serva, dithiothreitol from

Calbiochem and ATP disodium salt from Boehringer, Mannheim. Penicillin N, isopenicillin N, DAOC and DAC were supplied by Ciba-Geigy AG.

Organism, Media and Culture Conditions

Well grown agar slant cultures of an industrial strain of *Cephalosporium acremonium* (synonym: *Acremonium chrysogenum*) TR 4 were inoculated into 100 ml of vegetative medium 154A in 500-ml flasks. The flasks were incubated for 4 days at 25°C on a rotary shaker (250 rpm). 7.4 liters of production medium ZEN 1, prepared as described below, were inoculated with 600 ml of the 4-day old vegetative culture. The fermentation was carried out in a 14-liter laboratory fermenter (Chemap AG, Männedorf, Switzerland). The fermentation was held at a temperature of 26°C and an overpressure of 0.8 bar. The culture was agitated at 800 rpm with a flat bladed turbine and the air flow rate was 1 vol/vol/minute. During the first 78 hours a total of 1,290 g glucose was fed as a 70% solution according to a preset program. From 78 hours onward soya oil was added at a rate of 1.0 ml/liter/hour. Cells were harvested after 112 hours.

The vegetative medium 154A contained (g/liter): peptone (Difco) 10.0, malt extract (Difco) 24.0, yeast extract (Difco) 26.8, $CaCO_3$ 5.0, soya oil 2.5, tap water. The pH after sterilization (20 minutes, 120°C) was 7.3.

Preparation of 1 liter of fermentation medium ZEN 1: 40 g corn steep powder and 10 g peanut meal were suspended in 750 ml tap water. The pH was adjusted to 6.2. After heating for 60 minutes at 100°C, the suspension was centrifuged for 10 minutes at $4,000 \times g$. To the supernatant, the following nutrients were added in g: $(NH_4)_2SO_4$ 12.0, urea 3.0, $MgSO_4 \cdot 7H_2O$ 7.0, K_2SO_4 5.0, $CaCl_2$ 0.3, $FeSO_4 \cdot 7H_2O$ 0.1, $MnSO_4 \cdot H_2O$ 0.038, $ZnSO_4 \cdot 7H_2O$ 0.025, $CuSO_4 \cdot 5H_2O$ 0.025, Cerelose 8.0, soya oil 10.0, antifoam agent (SAG 471) 0.6, tap water. The volume was made up to 1 liter. Sterilization of the medium was carried out in the fermenter.

Preparation of Cell-free Extract

Mycelium was recovered from the fermentation broth by centrifugation and washed twice with cold deionized water. 10 g wet cells were suspended in 20 ml ice-cold buffer (0.05 M Tris-HCl, pH 8.0, 0.01 M KCl, 0.01 M $MgSO_4$) and added to 20 g precooled glass beads (ϕ 0.45~0.50 mm) in a 90-ml glass tube. Cells were ruptured by vibrating the mixture with an immersed glass piston attached to a Vibromixer (Chemap, Typ E 1) for 1 minute (frequency 50 Hz). Cell debris was removed by centrifugation at $15,000 \times g$ for 10 minutes. The supernatant was filtered successively through a 0.6 μm and then a 0.2 μm MF-Millipore filter. This filtrate A was stored at −60°C. All the operations described were carried out at 0~4°C.

Purification of the Enzyme

Ammonium Sulfate Precipitation: The protein precipitation from filtrate A, obtained between 35% and 80% $(NH_4)_2SO_4$ saturation, was collected by centrifugation and dissolved in buffer I (0.05 M Tris-HCl, pH 7.4, 0.01 M KCl, 0.01 M $MgSO_4$). This was designated enzyme solution B.

Column Chromatography: Enzyme solution B was adsorbed on to a Sephadex G-75 column (2.2×50 cm), equilibrated and then eluted with buffer I. The active fractions were pooled and precipitated with 80% $(NH_4)_2SO_4$. The precipitate was collected by centrifugation and the proteins were dissolved in buffer II (0.02 M Tris-HCl, pH 7.3, 0.05 mM DAOC, 0.1 mM dithiothreitol). After desalting on Sephadex G-25, this solution C was chromatographed on a column (1.5×8.0 cm) of DEAE-Trisacryl M, equilibrated with buffer II. Protein was eluted from the column at a flow rate of 12 ml/hour with a linear gradient (0.02~0.3 M Tris-HCl in 120 ml) of buffer II. The most active fractions were pooled and concentrated by ultrafiltration through a DDS GR 81 P membrane at 5 bar. This enzyme solution D was stored at −60°C.

Chromatofocusing: Enzyme solution B was loaded on to Sephadex G-25, equilibrated with buffer III (0.025 M histidine-HCl, pH 6.2, 0.05 mM DAOC, 0.1 mM dithiothreitol) and then eluted with the same buffer. This solution was chromatographed on a column (1.0×25.0 cm) of polybuffer exchanger PBE 94 (Pharmacia), equilibrated with buffer III. After adsorption, the proteins were eluted at a flow rate of 17 ml/hour with buffer IV (polybuffer 74 - HCl diluted with distilled H_2O, 1: 8, pH 4.0, 0.05 mM DAOC, 0.1 mM dithiothreitol). Protein peaks were monitored at 280 nm.

Electrofocusing: The isoelectric focusing (IEF) was performed on a cooled Pharmacia flat-bed electrofocusing unit. Enzyme solution D was focused in a 5% polyacrylamide gel of 0.5 mm thickness (LKB), containing 3% (w/v) Ampholines, ranging from 4.0 to 6.5 pH units. After focusing in a lengthwise direction for 3 hours at 800 V, the band containing the enzyme activities was cut out and immediately laid on a second gel of the same kind. Focusing at 800 V was performed for 4 hours. Gel fragments were cut out along the pH gradient and eluted into buffer I overnight. This enzyme solution E was tested for enzyme activity. All the operations described were carried out at 4~10°C. The gels were stained with Coomassie Brilliant Blue R-250 according to LKB instructions for analytical electrofocusing on polyacrylamide gels.

SDS-Polyacrylamide Gel Electrophoresis (PAGE): Enzyme solution E was subjected to electrophoresis under denaturing conditions, according to LAEMMLI[17]. The polyacrylamide concentration was 12%. After fixation the gel was stained by the Bio-Rad Silver Stain technique.

Assays

Dioxygenase: To measure DAOC-synthetase activity, a reaction mixture was prepared according to KUPKA *et al.*[9] except that the penicillin N concentration was reduced to 0.1 mM. The amount of protein in the test ranged from 0.05 to 0.2 mg/ml, depending on the purification step. After 10 to 45 minutes, the time varying according to the enzyme activity, the reaction was stopped by adding ethanol (1:1). The precipitated proteins were removed by centrifugation and the supernatant analyzed by HPLC. DAOC-hydroxylase activity was analyzed under the same conditions as the DAOC-synthetase activity, except that DAOC was used as the substrate.

HPLC: A Zorbax BP-NH_2 (Dupont) column (4×250 mm) and a solvent system as described by MILLER *et al.*[18] were used. The pH of the solvent system was adjusted to 4.0. DAOC and DAC served as standards. 50 μl samples were injected and the absorption peaks were monitored at 262 nm (the amount of product formed was calculated with a program which integrated the area of the peaks).

Protein: Protein was determined according to BRADFORD[19]. Bovine serum albumin was used as the standard.

Results

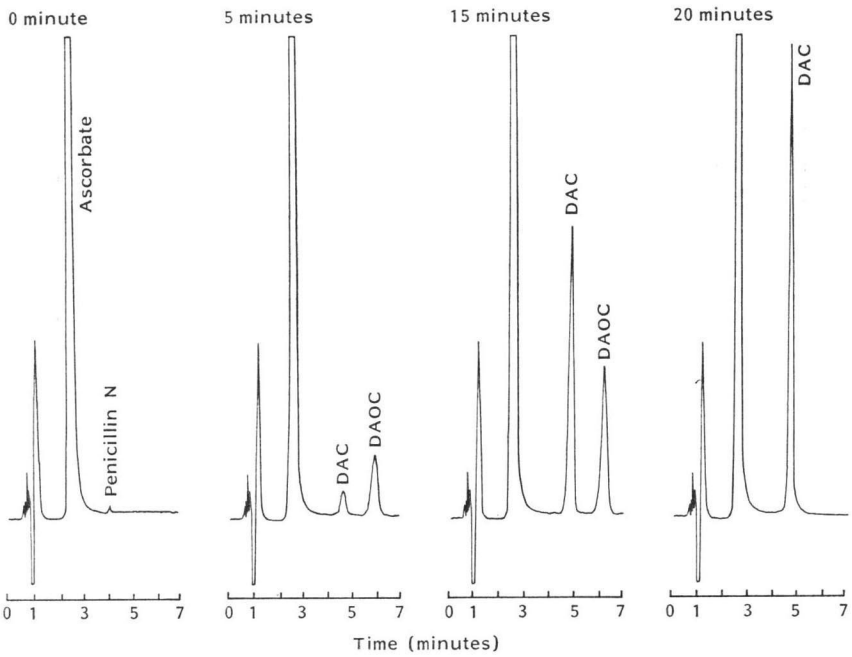
Cultivation

To facilitate separation of cells from the medium components and obtain cell extracts with high activities of the enzymes to be studied, a solid-free medium and a cultivation procedure were developed which allowed simulation of industrial production conditions on laboratory scale. In the course of the fermentation, the non-productive filamentous vegetative mycelium transformed into thick, bulging arthrospores[20]. These cells which produced antibiotic at the maximum rate were harvested at 112 hours, when the cephalosporin C titer was about 10 g/liter.

Product Analysis

DAOC-synthetase/hydroxylase activity was determined by HPLC analysis. By selecting a pH of 4.0 for the elution buffer instead of 2.6 as used by MILLER *et al.*[18], the performance of the HPLC-system could be improved. By this means the time required to analyze a sample was reduced from 10 to 6 minutes without losing to much resolution efficiency. Incubation of penicillin N with crude and partially purified cell extract gave rise to two products. Their identity with DAOC and DAC was confirmed in three ways. Both products were shown to be β-lactams by their sensitivity to β-lactamase P-99[21]. The UV spectra obtained by UV-range scanning of the peaks and the retention times on HPLC were identical with those of the reference standards DAOC and DAC. When penicillin N was used as a substrate first DAOC and then DAC were formed. In the presence of higher amounts of enzyme, all the DAOC present was transformed into DAC within 20 minutes (Fig. 1). With DAOC as the substrate

Fig. 1. HPLC chromatograms of samples taken at different reaction times.



only DAC was formed.

Cell Rupture and Enzyme Purification

Since DAOC-synthetase is unstable and cell breakage by sonication gives rise to local heat production, cells were ruptured with a Vibromixer. The optimal vibration time of 1 minute led to an active enzyme extract. About 70% of the cells were destroyed without any significant (<1°C) heat production.

Since purification yields of DAOC-synthetase/hydroxylase were low and could not be determined during IEF, no data on yield and degree of purification are shown. DAOC-synthetase and DAOC-hydroxylase activities could not be separated by means of fractional $(NH_4)_2SO_4$ precipitation and Sephadex G-75 gel chromatography. A further attempt to separate the two activities was made by using ion exchange chromatography on DEAE-Sepharose CL-6B. However, the recovery yield was too low to carry out further purification. In an attempt to improve yields the ion exchanger DEAE-Trisacryl M was tried. Proteins were adsorbed in 0.02 M Tris buffer II, pH 7.3 and eluted by increasing the buffer concentration linearily to 0.3 M. Dithiothreitol and DAOC were included in both buffers to increase stability of the enzyme. DAOC-synthetase and DAOC-hydroxylase activities eluted together shortly after the 3 main protein peaks (Fig. 2). Additional purification was obtained by IEF. Enzyme solution D was focused and revealed a main band at a pH of 4.6 ± 0.1, which showed activity for both reactions. Since low voltage was used to preserve the enzyme, interference from other proteins still occurred. To obtain a better separation, the proteins from the gel with the highest enzyme activities were rerun on a second gel. DAOC-synthetase and DAOC-hydroxylase activities were again present in one band at a pH of 4.6 ± 0.1 (Fig. 3). Additional determination of the pI by chromatofocusing of the enzyme solution C on a polybuffer exchanger PBE 94 column gave a value of 4.5 ± 0.1.

The purity of the main active band after the second IEF was determined by SDS-PAGE. By the

Fig. 2. Purification of DAOC-synthetase/hydroxylase by ion exchange chromatography on DEAE-Trisacryl M.

Formation of DAOC (■) by DAOC-synthetase and of DAC (□) by DAOC-hydroxylase. Reaction time; 35 minutes. Substrate concentration; 0.1 mM.

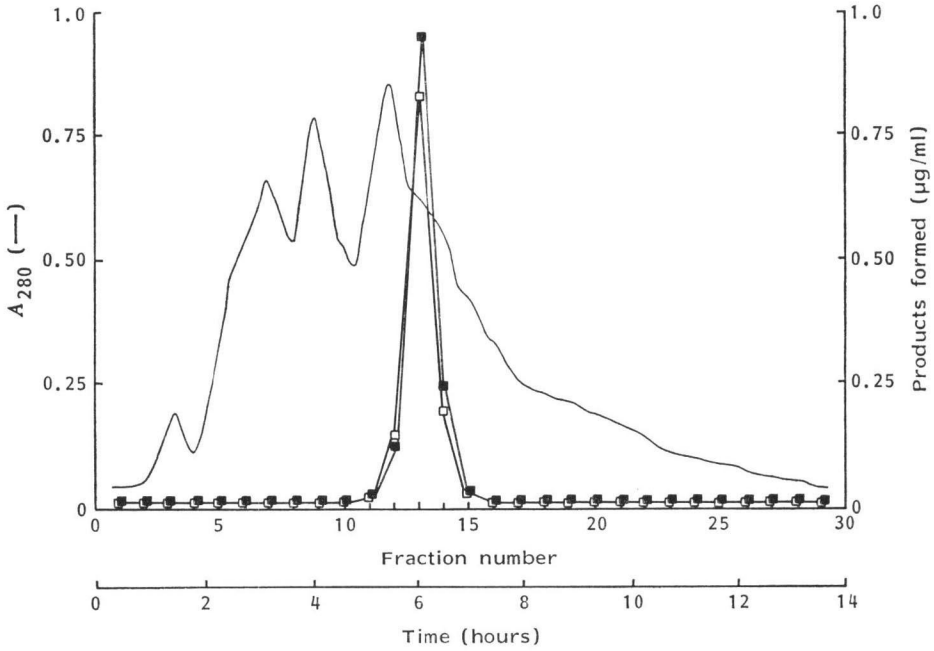
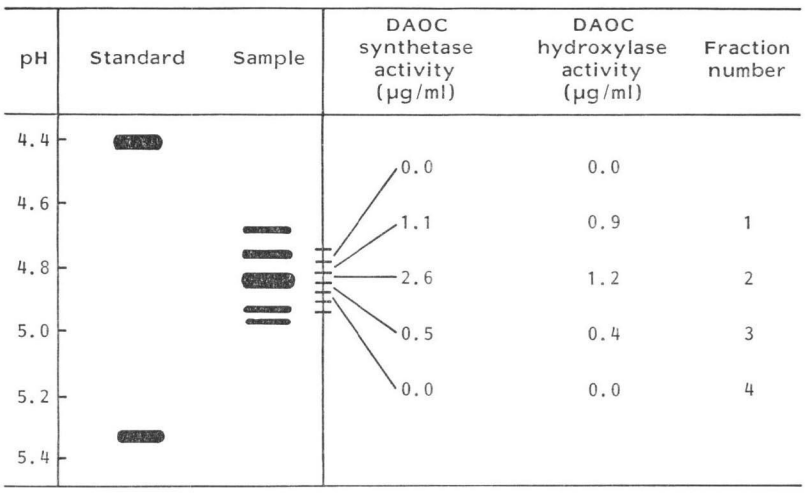
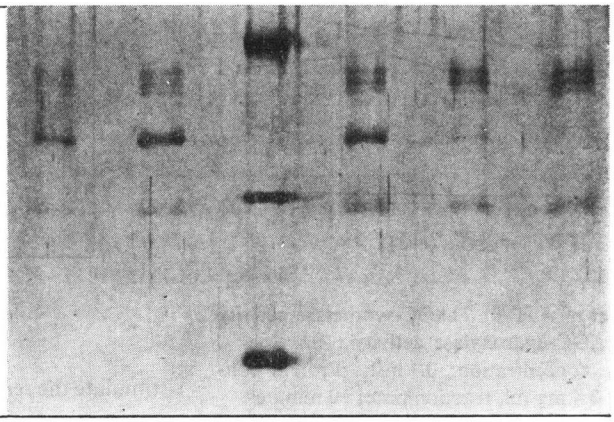


Fig. 3. Drawing of the stained polyacrylamide gel after the second focusing. DAOC-synthetase/hydroxylase activities of the cut out bands are shown.



sensitive silver stain technique several bands could be detected (Fig. 4). The only one which correlated with both enzyme activities (determined after second IEF) was at a molecular weight of 33,000±2,000. In the fractions, where this main band was lacking, no activity could be found. Based on this finding we conclude tentatively that the ring expansion of penicillin N and the oxygenation of DAOC are catalyzed by the same enzyme.

Fig. 4. SDS-PAGE of the active band and adjacent areas after second IEF.
Activities were determined after the second IEF.



	1	2	3	4	5
DAOC synthetase activity (μg/ml)	1.1	2.6	0.5	0	0
DAOC hydroxylase activity (μg/ml)	0.9	1.2	0.4	0	0

Fig. 5. Time slope of product formation of DAOC-synthetase/hydroxylase.
(■) DAOC-synthetase activity. (□) DAOC-hydroxylase activity.
Protein concentration; 0.4 mg/ml.

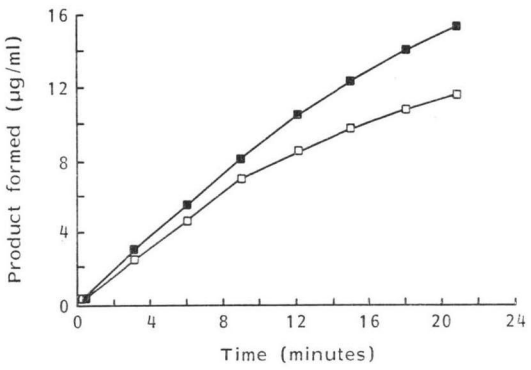
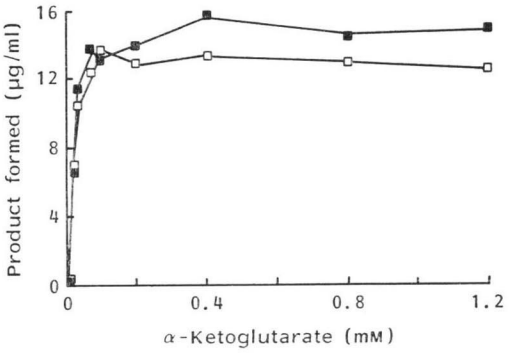


Fig. 6. Effect of α-ketoglutarate on DAOC-synthetase activity (■) and DAOC-hydroxylase activity (□).
Substrate concentration; 0.1 mM. Protein concentration; 0.4 mg/ml, reaction time; 10 minutes.



Catalytic Properties

Cofactor dependence and oxygen requirement were determined for the first time under identical test conditions for both reactions, ring expansion and oxygenation. Since only a small amount of enzyme was available after ion exchange chromatography and IEF, the catalytic properties of DAOC-synthetase/hydroxylase had to be determined with enzyme solution C. Reaction times of less than 10 minutes were chosen because the reaction rate after 10 minutes ceased to be linear with time (Fig. 5). Preincubation of the reaction mixture without substrate, as described by TURNER *et al.*[12], did not further

Fig. 7. Effect of $FeSO_4$ on DAOC-synthetase activity (■) and DAOC-hydroxylase activity (□).
Substrate concentration; 0.1 mM. Protein concentration; 0.4 mg/ml, reaction time; 10 minutes.

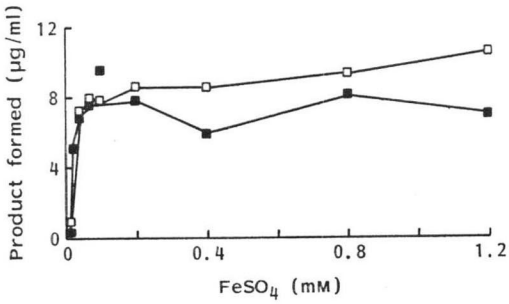


Fig. 8. Effect of ascorbate on DAOC-synthetase activity (■) and DAOC-hydroxylase activity (□).
Substrate concentration; 0.1 mM. Protein concentration; 0.4 mg/ml, reaction time; 10 minutes.

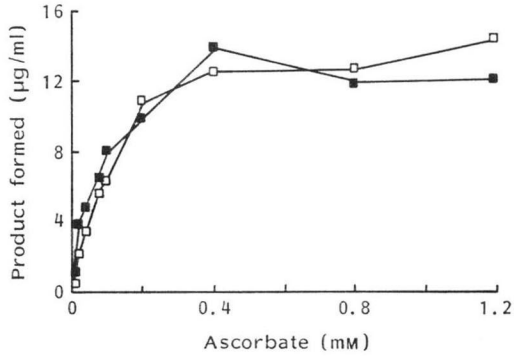
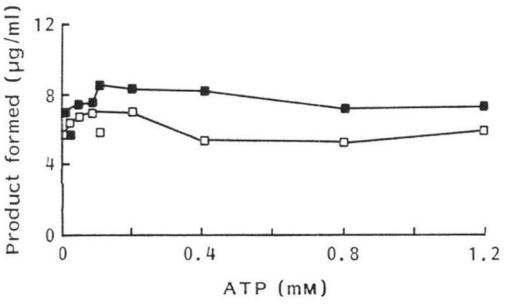


Fig. 9. Effect of ATP on DAOC-synthetase activity (■) and DAOC-hydroxylase activity (□).
Substrate concentration; 0.1 mM. Protein concentration; 0.4 mg/ml, reaction time; 10 minutes.



stimulate the reaction. The reason for this difference in findings is not known but it may be due to another enzyme purification procedure. The demand of both reactions for α-ketoglutarate and $FeSO_4$ is shown in Figs. 6 and 7. By omitting these two cofactors, no products were formed. The simultaneous dependence on α-ketoglutarate and $FeSO_4$ supports the hypothesis mentioned by WALSH[22] that an enzyme bound persuccinate intermediate is formed from an enzyme-Fe-O_2-α-ketoglutarate complex. Ascorbate, which is thought to reduce Fe^{3+}, is required for both activities (Fig. 8), whereas the addition of ATP shows only a slight stimulation of about 20~25% (Fig. 9). From several experiments a *Km* of 0.03 mM for penicillin N and of 0.02 mM for DAOC were obtained. When isopenicillin N was added as substrate to the IEF purified enzyme solution it was not converted to a cephalosporin.

Discussion

A characteristic of the DAOC-synthetase/hydroxylase is its instability during purification. When purifying DAOC-synthetase, KUPKA *et al.*[9] also observed a pronounced instability. Since alternative purification methods such as chromatofocusing, fast protein liquid chromatography and affinity chromatography on Sepharose linked α-ketoglutarate did not result in a significant improvement and no sufficiently good stabilizing agent was found, it was necessary to forego the final preparative purification and to work with a partially purified enzyme.

The purest active preparation obtained after repeated IEF contained mainly 3 bands on SDS-gel, 2 of which were also present in neighboring inactive fractions. Thus, the DAOC-synthetase/hydroxylase activities can most probably be attributed to a protein migrating as a single band in a SDS-gel and having a molecular weight of *ca.* 33,000. This molecular weight is in agreement with the value estimated by KUPKA *et al.*[9] for the DAOC-synthetase. Further indirect evidence in support of our conclusion that one enzyme catalyzes the ring expansion and oxygenation reactions is given by the following observations. At no point during the many different purification experiments was it possible to detect any

sign of separation of the two activities. The requirements for the cofactors α-ketoglutarate, $FeSO_4$ and ascorbate are virtually identical for both reactions. To our knowledge, extensive screening for DAOC overproducing *C. acremonium* mutants has not led to the discovery of strains with a block at the oxygenation step. The DAOC mutants described by FUJISAWA *et al.*[23] and QUEENER *et al.*[24] still produced considerable amounts of DAC. The same holds true for the mutants isolated in our laboratory. LIERSCH *et al.*[15] did in fact describe a mutant that only produced DAOC, but after testing the productivity of this strain in later years using a more sophisticated HPLC test system, the mutant was also shown to produce DAC and cephalosporin C. If ring expansion and oxygenation were to be catalyzed by two separate enzymes, one would expect it to be much easier to find a mutant blocked for only one of the enzymes. The cofactor requirements are typical for a dioxygenase and are in good agreement with what has been described so far in the literature[5,10,12] for the single activities. The only exception is the absence of a strong positive effect of ATP addition. Therefore, one cannot be sure if ATP is strictly necessary for these enzyme reactions, although it does have a slight stimulatory effect. However, it is possible that enzyme bound ATP assists in the two oxygenations which would then explain the minimal effect of additional ATP.

Although the net result of the ring expansion reaction shows no incorporation of oxygen, the actual mechanism may involve an oxygenation of penicillin N to its sulfoxide[25,26]. This may bring about the opening of the ring structure followed by closure to the six-membered ring and concomitant release of the hydroxyl group. If this assumed mechanism were true both the ring expansion and the oxygenation reactions could be considered as oxygenations involving the most reactive group present in the substrate molecule. The specificity of the enzyme seems to be directed more towards the side chain than to the β-lactam nucleus. The L-configuration of the α-aminoadipic acid moiety of isopenicillin N prevents ring expansion. An interesting reaction in which oxygenation was also observed, was recently described by ADLINGTON *et al.*[27]. A crude extract of *C. acremonium* mycelium catalyzed the conversion of 3-exomethylene cephalosporin C to DAC. No formation of DAOC as an intermediate was observed. Direct hydroxylation to DAC by the same enzyme as that which converts penicillin N to DAOC and DAOC to DAC may occur. Support for this hypothesis is given by the fact that no 3-exomethylene cephalosporin C could be found in *C. acremonium*.

There is now sufficient scientific data on DAOC-synthetase/hydroxylase to conclude that different control mechanisms are responsible for the amount of enzyme produced and its activity during the course of the fermentation. A high glucose level in the fermentation medium (6%) strongly represses ring expansion activity, but cyclization and subsequently penicillin N production is only slightly repressed[28~31]. The decreased activity of the oxygen dependent DAOC-synthetase/hydroxylase might be also due to an intracellular oxygen deficiency, since in the presence of large amounts of glucose, the oxygen requirements for glycolysis and respiration are high. This would agree with our finding that DAOC-synthetase/hydroxylase activities can be strongly inhibited *in vitro* by excluding oxygen. A more thorough understanding of the factors governing synthesis and activity of the two enzymes DAOC-synthetase/hydroxylase and DAC-acetylase is necessary. High activities of both enzymes are a prerequisite to prevent excretion of cephalosporin C precursors and to ensure maximal cephalosporin C productivity.

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