Purification of Acetyl Coenzyme A: Deacetylacephalosporin C O-Acetyltransferase from Acremonium chrysogenum

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Received February 21, 1992

Acetyl CoA: deacetylacephalosporin C O-acetyltransferase, which catalyzes the final step of the biosynthetic pathway to cephalosporin C, was stabilized by a buffer solution containing 7-aminocephalosporanic acid and purified over 1300-fold from Acremonium chrysogenum. The purified enzyme has a molecular weight of 55,000 as measured by gel filtration. SDS-polyacrylamide gel electrophoresis showed two subunit bands corresponding to molecular weights of 27,000 and 14,000. The enzyme has an isoelectric point at pH 4.0 and optimum activity at pH 7.5.

Several enzymes involved in the biosynthetic pathway of cephalosporin C in Acremonium chrysogenum have been purified and the corresponding genes studied by cloning. Acetyl CoA: deacetylacephalosporin C O-acetyltransferase (DCPC-ATF) is important in catalyzing the final step to cephalosporin C and therefore has been the subject of extensive study. The DCPC-ATF activity was first reported by Fujisawa et al. in cell-free extracts from Acremonium chrysogenum. Subsequently the enzyme has been partially purified by Scheidegger et al., further purification and characterization have been held up by its extreme instability and the enzyme has been left poorly understood. In this communication, we describe our studies on the stabilization and purification of DCPC-ATF. Studies with the purified enzyme found two nonidentical subunits and their N-terminal amino acid sequences, which might pave the way to investigation of the gene structure of this enzyme.

Materials and Methods

Organism, culture conditions, and preparation of cell-free extracts. Acremonium chrysogenum IS-5 (Komatsu et al.) was grown at 25°C for 4 days in a medium consisting of the following: 2% saccharose, 0.5% calcium carbonate, 3% starch, 5% molasses, 6% defatted soybeans, and 3% methyl oleate (pH 6.4). Cells were harvested by centrifugation (6000 x g), washed with 50 mM phosphate buffer (pH 7.0) containing 0.85% sodium chloride, and resuspended in 50 mM phosphate buffer (pH 7.0) or buffer containing ATF-BASE [10 mM dithiothreitol, 10 mM 7-aminocephalosporanic acid (7-ACA), 1 mM p-amidinophenacylmethane-sulfonilfluoride hydrochloride, 1 mM EDTA, and 15% ethylene glycol]. Then the cells were disrupted by ultrasonic treatment (5 min at 5°C) and centrifuged (15,000 x g, 10 min) to obtain a supernatant fraction as a cell-free extract.

Enzyme assay. The DCPC-ATF assay mixture (0.1 ml) contained 0.1 M Tris-HCl (pH 7.5), 4 mM magnesium sulfate, 2.5 mM deacetylacephalosporin C (DCPC), 5 mM acetly CoA, and 0.0001 to 0.001 U of the enzyme. It was incubated at 30°C for 30 min, then ethanol (0.1 ml) was added to stop the reaction. The mixture was centrifuged at 10,000 x g for 2 min to obtain a supernatant. The cephalosporin C in the supernatant was measured by a high performance liquid chromatography system, using a ZORBAXRP NH2 column (4.5 mm x 25 cm, E. I. Du Pont De Nemours and Company, U.S.A.) with a solution of 4% acetic acid, 4% methanol, 8% acetonitrile, and 84% water, at a flow rate of 1.5 to 2.2 ml/min, and the wavelength for measurement was 254 nm. One unit of DCPC-ATF activity was defined as catalyzing the formation of 1 μmol of cephalosporin C per min under the standard assay conditions. Protein was measured by the method of Bradford with bovine serum albumin as a standard.

Stabilization of crude enzyme. The stability of the enzyme in cell-free extracts was examined by incubating the preparation at 5°C in the presence or absence of different concentrations of reagents as potential stabilizers. After incubation for various lengths of time, the enzyme reactions were done to measure the residual activity.

DCPC-ATF purification. The following operations were done at 5°C. A cell-free extract was prepared from 500 g of the wet preparation of cells by this procedure using the ATF-BASE buffer and found to have a total protein of 22.9 g and total activity of 25.7 U. One-fourth volume of the cell-free extract was put onto a DEAE-Sepharose CL-6B column (4 x 50 cm, Pharmacia Fine Chemicals, Sweden) which had been equilibrated with the ATF-BASE buffer, and elution was done with a linear gradient of 0 to 0.5 M sodium chloride in the ATF-BASE buffer (total volume; 2500 ml). The DCPC-ATF fraction was eluted at 0.17 to 0.2 M sodium chloride. This procedure was repeated 4 times to treat the whole volume of the crude enzyme solution, and the active fractions were pooled. To the pooled fraction, ammonium sulfate was added to 25% saturation, and the resultant solution was put onto a Phenyl-Sepharose CL-4B column (3 x 20 cm, Pharmacia Fine Chemicals, Sweden) which had been equilibrated with the ATF-BASE buffer. Elution was done with a linear gradient of 25 to 0% ammonium sulfate and 10 to 0% ethylene glycol in the ATF-BASE buffer, the flow rate was 150 ml/hr, and the total volume was 1000 ml. The active fractions, eluted at 8 to 3% ammonium sulfate and 40 to 48% ethylene glycol, were pooled and concentrated with a Centriprep 10 (Amicon Plastics Inc., U.S.A.) to a volume of 6 ml. One-third of the concentrated solution was packed onto a Sephacryl S-200 Superfine column (4 x 80 cm, Pharmacia Fine Chemicals) which had been equilibrated with the ATF-BASE buffer, and gel filtration was done three times at a flow rate of 40 ml/hr. Thus active fractions were pooled and concentrated with the Centriprep 10 to a volume of 2 ml. The concentrated solution was put onto a TSK-gel DEAE-SPW column (2.1 x 15 cm, Toyoda Soda Mfg. Co., Ltd., Japan) which had been equilibrated with the ATF-BASE buffer and eluted with a linear gradient of 0 to 0.5 M sodium chloride in the ATF-BASE buffer, a flow rate of 3 ml/min, and a total volume of 300 ml. The active fraction thus collected was found to have total protein of 0.1 mg and total activity of 0.13 U.

Molecular weight measurement. The molecular weight of active DCPC-ATF was measured by gel filtration using TSK gel G3000 SWXL (7.8 mm x 30 cm, Toyoda Soda Mfg. Co., Ltd.) which had been equilibrated with the ATF-BASE buffer. As the molecular weight standards, bovine serum albumin [molecular weight (MW): 67,000], ovalbumin (MW: 43,000), chymotrypsininogen A (MW: 25,000), and ribonuclease A (MW: 13,700) were employed.
**Results and Discussion**

**Stabilization of DCPC-ATF**

DCPC-ATF was very unstable when extracted from the cells in 50 mM phosphate buffer (pH 7.0), and the activity was lost in 16 hr at 5°C (Fig. 1). Potential stabilizers were examined, and the enzyme was found to be stable for 48 h in the presence of a combined mixture, designated ATF-BASE, of several agents described in Materials and Methods. The most effective component was 7-ACA, whose depletion from ATF-BASE greatly reduced the enzyme stability. It is suggested that 7-ACA exerts this effect by interacting with the enzyme as an acetyl donor, since deacetyl-7-ACA did not stabilize the enzyme and

![Fig. 1. In Vitro Stability of Crude DCPC-ATF Activity.](image)

Cell-free extract was incubated in 50 mM phosphate buffer (pH 7.5) supplemented with: none, ○; ATF-BASE, ●; ATF-BASE lacking 7-ACA, ▲.

![Fig. 2. Purification of DCPC-ATF on (A) DEAE Sepharose CL-6B, (B) Phenyl-Sepharose CL-4B, (C) Sephacryl S-200, and (D) TSK Gel DEAE-3PW Chromatographies.](image)
acetyl CoA was substituted for 7-ACA to some extent in the DCPC-ATF reaction (data not shown).

### Purification

The purification process is summarized in Table I. In the presence of ATP-BASE purification of the enzyme was done by weak anion exchange chromatography, hydrophobic interaction chromatography, gel filtration, and DEAE high performance liquid chromatography (Fig. 2). The enzyme was purified over 1300-fold to apparent homogeneity as suggested by its appearance on SDS-PAGE (Fig. 4B). The specific activity of the purified enzyme was 1.3 U/mg with a 0.5% yield from the cell extract. The enzyme has been partially purified to 15 fold by Scheidegger et al., but further purification and characterization have been held up by its extreme instability. This is the first report of the purification of DCPC-ATF to apparent homogeneity and a possible subunit structure as described below.

### Enzymatic properties

The DCPC-ATF activity was optimum at pH 7.5 under the standard assay conditions (Fig. 3). The molecular weight of the native enzyme was estimated to be 55,000 by gel filtration on the TSK-gel G3000 SWXL column (Fig. 4A). SDS-PAGE resolved two protein bands with molecular weights of 27,000 and 14,000, respectively (Fig. 4B). As described in another paper, the two bands were found to be nonidentical subunits of DCPC-ATF by cDNA cloning and designated subunits I and II. The cDNA information suggests that DCPC-ATF is synthesized as a single-chain precursor protein and then converted to the two constituent subunits of DCPC-ATF by proteolytic processing. The isoelectric point of the purified enzyme was pH 4.0, measured by chromatofocusing with PBE 94 (Pharmacia Fine Chemical). The enzyme followed Michaelis-Menten kinetics, and the $K_m$ for acetyl CoA and DCPC were 1.05 mM and 0.29 mM, respectively. The effect of Mg$^{2+}$ on DCPC-ATF activity reported by Fujisawa et al. was not observed (data not shown).

### Amino acid sequences

The subunit proteins and polypeptide fragments generated by partial digestion of purified DCPC-ATF were analyzed by N-terminal amino acid sequencing, and the results are shown in Table II. No homology was found between the disclosed N-terminal sequences of the two subunits. The sequences presented here have provided an essential tool to clone the DCPC-ATF gene, as described in another paper.

### References