Isolation of pure anhydrotetracycline oxygenase from *Streptomyces aureofaciens*

Ivana VANČUROVÁ,*† Jindřich VOLC,* Miroslav FLIEGER,* Jiří NEUŽIL,* Jana NOVOTNÁ,* Jaromír VLACH† and Vladislav BĚHAL*

*Institute of Microbiology, Czechoslovak Academy of Sciences, Vídeňská 1083, 142 20 Prague 4, and

†Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 160 00 Prague 6, Czechoslovakia

Anhydrotetracycline oxygenase was purified to homogeneity from *Streptomyces aureofaciens*, a producer of tetracycline. The enzyme was purified 60-fold in a 40 % yield by a two-step procedure using a combination of hydrophobic chromatography and ion-exchange h.p.l.c. Purified anhydrotetracycline oxygenase was homogeneous according to SDS/polyacrylamide-gel electrophoresis, isoelectric focusing, ion-exchange h.p.l.c. on a Mono Q HR 5/5 column and size-exclusion h.p.l.c. on a TSK G 3000 SW column. The enzyme consists of two subunits of M_r 57 500, as determined by SDS/polyacrylamide-gel electrophoresis.

INTRODUCTION

Among the enzymes participating in the biosynthesis of tetracyclines, three have been described so far: *S*-adenosylmethionine:dedimethylamino-4-amino-anhydrotetracycline *N*-methyltransferase (Miller & Hash, 1975a), anhydrotetracycline (ATC) oxygenase (Běhal *et al.*, 1979) and NADP:tetracycline 5a(11a)-dehydrogenase (Miller & Hash, 1975b; Erban *et al.*, 1985). None of them has as yet been isolated in pure state.

ATC oxygenase (EC 1.13.12.–) catalyses the penultimate reaction of the tetracycline biosynthetic pathway i.e. the conversion of ATC into dehydro-tetracycline (Scheme 1). It is a mono-oxygenase which requires for its catalytical function NADPH and atmospheric oxygen (Běhal *et al.*, 1979). It can hydroxylate anhydrochlortetracycline equally well as it does anhydrotetracycline. The enzyme has been partially purified and characterized by Běhal *et al.* (1983) and Vančurová *et al.* (1987). Its M_r is 115 000 and its pI is 5.3 (Vančurová *et al.*, 1987). The present paper describes the purification and subunit structure of ATC oxygenase. ATC oxygenase is thus the first enzyme of tetracycline biosynthesis to be isolated in a homogeneous form.

Scheme 1. Conversion of anhydrotetracycline into dehydro-tetracycline

EXPERIMENTAL

Materials

Anhydrotetracycline was prepared by a procedure described by Schlecht & Frank (1975). Phenyl-Sepharose CL-4B and Mono Q HR 5/5 prepacked h.p.l.c. columns were from Pharmacia Fine Chemicals, Uppsala, Sweden. Tris and Lubrol were from Sigma Chemical Co., St. Louis, MO, U.S.A. Glucose 6-phosphate and glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* were from Serva Feinbiochemicals, Heidelberg, Germany. NADP was from Reanal, Budapest, Hungary. All other chemicals were of the highest purity available.

Organism and culture conditions

Streptomyces aureofaciens 50/137, which was derived from the high-producing strain 84/25, obtained from the Research Institute of Antibiotics and Biotransformations, Roztoky, near Prague, was used. The cultivation was carried out in 500 ml Erlenmeyer flasks in a reciprocal shaker (1.6 cycles/s, 28 °C). Soya-bean meal medium used for submerged cultivation contained (g/l): sucrose, 30; soya-bean meal, 30; NaCl, 5; CaCO₃, 4; (NH₄)₂SO₄, 2; molasses, 2; corn steep, 5; benzyl thiocyanate, 0.002. The pH was 6.9. Soya-bean meal was used in the form of an extract. Benzyl thiocyanate was added to the sterilized medium in the form of an aqueous solution before inoculation. Media used for preparing vegetative inoculum contained no benzyl thiocyanate. The vegetative inoculum (5 %; 24 h) was used for the inoculation of a production medium.

Preparation of cell-free extract

A 24 h mycelium was separated from the fermentation broth by centrifugation at 4000 *g* for 5 min, washed with distilled water, and centrifuged at 20 000 *g* for 30 min. The mycelium was disintegrated in a Biox X-Press

Abbreviation used: ATC, anhydrotetracycline.

† To whom all correspondence should be addressed.

(LKB, Stockholm, Sweden), at -25°C and a pressure of 300 MPa. Broken mycelium was suspended in 0.1 M-Tris/HCl buffer, pH 7.4, and after extraction for 40 min the suspension was centrifuged for 30 min at 22000 g.

Purification of ATC oxygenase

Hydrophobic-interaction chromatography was performed on a phenyl-Sepharose CL-4B column (2.6 cm $\times$ 8.0 cm) at 20°C . The cell-free extract (150 ml) was supplemented with KCl (final concn. 0.8 M), pH was adjusted to 7.4 with 0.5 M-acetic acid, and the whole volume was loaded on a phenyl-Sepharose bed previously equilibrated with buffer A (0.1 M-Tris/HCl, pH 7.4). After the column was washed with buffer A, the absorbed material was eluted with a linear gradient (440 ml) of 0–100% buffer B (0.02 M-Tris/HCl, 0.5% Lubrol, pH 7.4) at a flow rate of 3 ml/min, and 8 ml fractions were collected. The eluate was monitored for absorbance at 280 nm. ATC oxygenase activity was located by assaying 0.05 ml amounts of each fraction.

Ion-exchange (Mono Q HR 5/5) chromatography

ATC oxygenase-containing fractions from hydrophobic-interaction chromatography were pooled (56 ml) and transferred into buffer C (0.02 M-Bis-Tris/HCl, pH 6.5) in an Amicon model 52 UF cell, with PM-10 membrane (Amicon Co., Danvers, MA, U.S.A.). Samples (8 ml; 12 mg of protein each) were applied via a 10 ml Superloop on to a Mono Q column equilibrated with buffer C. After the column was washed with the same buffer, the elution was continued with a linear gradient of 0–40% buffer D (1 M-NaCl in buffer C), and the eluate was monitored at 280 nm. Fractions (0.5 ml) were collected at a flow rate of 1 ml/min. The chromatography was done at 20°C .

Biochemical assays

The ATC oxygenase activity, measured as a decrease in ATC absorbance at 440 nm, was determined as described by Běhal *et al.* (1979). The reaction mixture (0.1 M-Tris/HCl buffer, pH 7.4, 0.04 mM-NADP⁺, 0.15 mM-glucose 6-phosphate, 2 μl of glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*/ml, ATC oxygenase sample) was saturated with O₂ and preincubated at 28°C . After temperature equilibration, 20 μM -ATC was added and the rate of decrease of absorbance at 440 nm was measured.

Proteins were determined by an absorbance method (Whitaker & Granum, 1980) and by the method of Lowry *et al.* (1951) with bovine serum albumin as

standard. All spectrophotometric measurements were done with a Cary 118 (Varian) spectrophotometer.

SDS/polyacrylamide-gel electrophoresis

This was conducted by the procedure of Laemmli (1970) in 10% polyacrylamide gels (90 mm $\times$ 180 mm $\times$ 1 mm thick) containing 0.1% SDS. Gels were run at a 170 V constant voltage until the Bromophenol Blue dye front was within 1 cm of the bottom of the gel (about 3.5 h). Gels were stained for protein with Coomassie Blue R-250 in methanol/acetic acid/water (5:1:4, by vol.). The same solution without the dye was used for destaining. Colour densities of the protein bands were measured with a densitometer (CS-930; Shimadzu, Kyoto, Japan).

The M_r of enzyme subunits was estimated from the relative mobilities of standard proteins. These were carbonic anhydrase (M_r 29000), ovalbumin (M_r 45000), bovine serum albumin (M_r 66000) and phosphorylase b (M_r 97400), from Sigma.

Isoelectric focusing

Analytical isoelectric focusing was performed as described by Kinzkofer & Radola (1981) on a Pharmacia FBE 3000 flat-bed apparatus, with polyacrylamide gels 50 μm thick. The V-h product was 1800 V-h, and maximum voltage was 2000 V. The Ampholine range was pH 3.5–10, and the Pharmacia broad-pI kit was used to calibrate the gel.

Size-exclusion h.p.l.c.

Gel filtration of the purified ATC oxygenase was performed with 0.02 M-Tris/HCl buffer, pH 7.4, on a TSK G 3000 SW column (7.5 mm $\times$ 300 mm) (LKB, Stockholm, Sweden), at a flow rate of 0.5 ml/min. The eluate was monitored for absorbance at 226 nm.

RESULTS AND DISCUSSION

Isolation of ATC oxygenase from a cell-free extract of *S. aureofaciens* was performed with a 24 h-old mycelium grown in the presence of benzyl thiocyanate, which causes a more than 2-fold increase in the activity of ATC oxygenase in a high-production strain (Běhal *et al.*, 1982). The specific activity of ATC oxygenase in the cell-free extract was 120 pkat/mg of protein. The results of purification of ATC oxygenase are summarized in Table 1.

The first purification step involved the use of hydrophobic chromatography on phenyl-Sepharose as

Table 1. Purification of ATC oxygenase

Purification step	Total volume (ml)	Total protein (mg)	Total activity (pkat)	Specific activity (pkat/mg of protein)	Purification (fold)	Recovery (%)
Crude extract	150	945	114000	120	1	100
Phenyl-Sepharose	56	84	82320	980	8.2	72
Ultrafiltration	53.8	80.6	66662	827	6.9	58
Mono Q chromatography	6.7	6.7	49258	7330	61.1	43

Fig. 1. Purification of ATC oxygenase by ion-exchange chromatography on a Mono Q HR 5/5 column

(a) ATC oxygenase was purified on a Mono Q HR 5/5 column under the following conditions: mobile phase, linear ionic-strength gradient of 0.02 M-Bistris/HCl with 1 M-NaCl, pH 6.5; temperature 20°C; flow rate 1 ml/min; detection at 280 nm (—); 0.5 ml fractions were collected and assayed for ATC oxygenase activity (●). (b) Active fractions (21 and 22) were desalted and rechromatographed.

described previously (Vančurová *et al.*, 1987). This method permits the application of a large amount of protein (~1 g) and provides a relatively high yield of ATC oxygenase activity (72%) and a high degree of purification (8.2-fold). Subsequent ultrafiltration, combined with buffer change, was accompanied by a minor decrease in the specific activity of ATC oxygenase (about 15%).

The next purification step was ion-exchange h.p.l.c. on a Mono Q HR 5/5 column (Fig. 1a). ATC oxygenase was eluted with 0.2 M-NaCl. This step resulted in a further 8.9-fold purification, with a 74% yield.

The overall purification of ATC oxygenase in the two steps was thus 61-fold, with a 43% yield. As the purification of ATC oxygenase on phenyl-Sepharose

entails a separation of a low- M_r factor (Vančurová *et al.*, 1987), the activity of the enzyme was always measured after activation with a heat-denatured cell-free extract, which exhibited no ATC oxygenase activity but increased the activity of purified ATC oxygenase up to 7-fold. However, it might be that the activation of ATC oxygenase by deproteinized cell-free extract does not include a full regeneration of the enzyme activity, and hence the actual purification could be higher.

The progress of purification was monitored by SDS/polyacrylamide-gel electrophoresis (Fig. 2). When subjected to SDS/polyacrylamide-gel electrophoresis after ion-exchange chromatography on a Mono Q HR 5/5 column, ATC oxygenase exhibited a single protein band corresponding to M_r 57 500, as determined by

Fig. 2. SDS/polyacrylamide-gel electrophoresis of ATC oxygenase samples at various stages of purification

Colour densities (A_{590}) of protein bands (stained with Coomassie Blue) are shown from: trace 1, cell-free extract; trace 2, phenyl-Sepharose fraction; trace 3, Mono Q fraction. ATC oxygenase is denoted by an arrow.

comparison with protein markers of known M_r . The M_r of ATC oxygenase determined by gel filtration on Sephadex G-200 SF is 115000 (Vančurová *et al.*, 1987), so the enzyme can thus be considered to be a dimer composed of two subunits of identical M_r . ATC oxygenase is the enzyme involved in secondary metabolite formation, i.e. in tetracycline biosynthesis. Since these enzymes are assumed to be present in the producer cell in low concentrations, we tried to confirm the homogeneity of 60-fold-purified ATC oxygenase by several independent methods. Fig. 1(b) shows the re-

Fig. 3. Size-exclusion h.p.l.c. of ATC oxygenase on a TSK G 3000 SW column

The ATC oxygenase from the Mono Q column was chromatographed. The eluate was monitored at 226 nm.

Fig. 4. Analytical isoelectric focusing of ATC oxygenase purified on a Mono Q HR 5/5 column

Lane 1 contained pI marker proteins. These were amyloglucosidase (pI 3.50), soya-bean trypsin inhibitor (pI 4.55), β -lactoglobulin A (pI 5.20), bovine carbonic anhydrase B (pI 5.85), human carbonic anhydrase B (pI 6.55), horse myoglobin (acidic band) (pI 6.85), horse myoglobin (basic band) (pI 7.35), lentil lectin (acidic band) (pI 8.15), lentil lectin (middle band) (pI 8.45), lentil lectin (basic band) (pI 8.65) and trypsinogen (pI 9.30), from Pharmacia. Lane 2 contained the purified ATC oxygenase (10 μ g) and lane 3 the crude cell-free extract (50 μ g).

chromatography of ATC oxygenase on a Mono Q HR 5/5 column under conditions identical with those used in its isolation. This step no longer increased the degree of purification (from 61.1-fold to 63.8-fold). Likewise, size-exclusion h.p.l.c. of homogeneous ATC oxygenase on a TSK G 3000 SW column yielded a single peak (Fig. 3). Analytical isoelectric focusing (Fig. 4) again showed the presence of a single protein band, although the ATC oxygenase was applied in large amount (10 μ g of protein).

Successful isolation of pure ATC oxygenase (as documented by four different methods) and its known localization on electrophoretograms will facilitate future study of the enzyme *in vivo*.

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