Purification and characterization of cephalosporin 7 α -hydroxylase from *Streptomyces clavuligerus*

Xinfa XIAO,*† Saul WOLFE† and Arnold L. DEMAIN*§

*Fermentation Microbiology Laboratory, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, U.S.A., and †Department of Chemistry, Simon Fraser University, Burnaby, B.C. V5A 1S6, Canada

Cephalosporin 7 α -hydroxylase, which catalyses the conversion of cephalosporins into their 7 α -hydroxy derivatives, was purified nearly 390-fold from *Streptomyces clavuligerus* through ion-exchange chromatography, (NH₄)₂SO₄ fractionation, gel filtration and dye chromatography, with the use of h.p.l.c. to monitor enzyme activity. The nearly pure enzyme migrates as a single major band, with an M_r of 32000 in SDS/PAGE. Its optimum pH is in the range 7.3–7.7. Under our conditions the reaction was fastest at temperatures in the range 20–30°C. The K_m for cephalosporin C is 0.72 mM, and the V_{max} is 15.4 μ mol of cephalosporin C hydroxylated/min per mg. Cephalosporin 7 α -hydroxylase did not show any deacetoxycephalosporin C synthase or deacetoxycephalosporin C hydroxylase activity.

INTRODUCTION

Methoxylation of cephalosporins (I) at the 7 α -position by *Streptomyces clavuligerus* proceeds in two steps: hydroxylation at C-7 to form 7 α -hydroxycephalosporin (II) and methylation of the hydroxylated intermediate to form 7 α -methoxycephalosporin (III) (Scheme 1) (O'Sullivan & Abraham, 1980; Hood *et al.*, 1983). When cephalosporin C (I, R = D- α -aminoadipyl, R' = acetoxy) was used as the substrate, the intermediate was 7 α -hydroxycephalosporin C (II, R = D- α -aminoadipyl, R' = acetoxy), which accumulated in the cell-free reaction mixture when this was incubated in the absence of S-adenosylmethionine, the methyl donor (Hood *et al.*, 1983). By using h.p.l.c. with a mobile phase modified from that used by Hood *et al.* (1983), the 7 α -hydroxycephalosporin C formed in the cell-free reaction mixture can be monitored directly (Xiao *et al.*, 1991). We have used this analytical method to follow the purification of cephalosporin 7 α -hydroxylase, and report here its almost complete purification and its characterization.

MATERIALS AND METHODS

Organism, medium and culture conditions

Streptomyces clavuligerus N.R.R.L. 3585 (A.T.C.C. 27064) was used in this work. The media and culture conditions were as described by Piret *et al.* (1990), except that the concentration of yeast extract in the seed medium was 0.05% instead of 0.1%.

Preparation and assay of cephalosporin 7 α -hydroxylase activity

The preparation and h.p.l.c. analysis of cell-free extracts were as described in our previous paper (Xiao *et al.*, 1991). Since synthetic 7 α -hydroxycephalosporin C was not available, 7 α -hydroxycephalosporin C formed in the cell-free reaction was quantified by the height of its h.p.l.c. peak, and this was used as a measure of cephalosporin 7 α -hydroxylase activity. A peak height of one small graduation of the chart per 15 min reaction time was regarded as the activity unit when recording at a range of 0.05. The specific activity of the enzyme is defined as activity units/mg of protein under the following conditions: 200 μ l of reaction mixture buffered with 100 mM-Mops/NaOH, pH 7.5; cofactors FeSO₄ (1 mM), L-ascorbic acid (1 mM) and sodium 2-oxoglutarate (1 mM), added together; reactions initiated by addition of substrate, cephalosporin C (237 μ g/ml), and conducted on a water-bath shaker (220 strokes/min for 15 min at 25°C); reactions terminated by addition of 10 μ l of acetic acid, followed by centrifugation in a Biofuge (Scientific Products, McGaw Park, IL, U.S.A.) (12000 g for 1.5 min) to remove precipitates before assay. Preliminary experiments indicated that the hydroxylation is linear with time for 15 min and also with respect to protein concentration in the range used in the reactions, i.e. 0.4–4 mg/ml for crude cell-free extract or 1.9–15.4 μ g/ml for purified cephalosporin 7 α -hydroxylase.

Protein assay

The protein contents of cell-free extracts and purified enzyme

Scheme 1. Pathway for the methoxylation of cephalosporins

† Permanent address: Antibiotic Research Institute, North China Pharmaceutical Corporation, Shijiazhuang, Hebei Province, People's Republic of China.

§ To whom correspondence should be addressed.

preparations were determined by the method of Bradford (1976), with BSA as standard.

Purification of cephalosporin 7 α -hydroxylase

Crude cell-free extract (150 ml) prepared from 1500 ml of fermentation broth was applied to an ion-exchange column (Sigma Chemical Co. DEAE-Sephacel column, 1.5 cm $\times$ 17 cm) equilibrated with 0.1 M-Mops/NaOH buffer, pH 7.5. The unbound proteins were removed by washing with 200 ml of the buffer. The enzyme was eluted with a linear gradient of KCl from 0 to 0.3 M in the buffer. Active fractions were pooled and fractionated by precipitation with (NH₄)₂SO₄. The protein that was precipitated in fractions of 35–60% saturation was collected by centrifugation (31000 *g* for 30 min at 4 °C), dissolved in 20 mM-Mops/NaOH buffer, pH 7.5, to a final volume of 2.5 ml, and subjected to gel-filtration chromatography (Pharmacia Sephacryl S200 column, 1.5 cm $\times$ 50 cm). The active fractions that were eluted with 20 mM-Mops/NaOH buffer, pH 7.5, were pooled and adjusted with 200 mM-MgSO₄ in 20 mM-Mops/NaOH buffer, pH 7.5, to a final Mg²⁺ concentration of 20 mM. The solution was applied to an Affi-Gel Blue column (1 cm $\times$ 2.5 cm) that had been equilibrated with 20 mM-Mops/NaOH buffer, pH 7.5, containing 20 mM-MgSO₄. The enzyme bound to the column was eluted with 20 mM-Mops/NaOH buffer, pH 7.5. Active fractions with specific activity of over 20000 units/mg (over 300-fold purification) were stored at –80 °C until used.

SDS/PAGE

Samples from each purification step were examined by SDS/PAGE, which was carried out in a 10% slab gel (0.75 mm thick) on a Midgel Electrophoresis Unit (Pharmacia LKB) at 40 mA starting current for each gel, essentially according to the Laemmli (1970) procedure. A set of *M_r* standards (Pharmacia) was used for the determination of the *M_r* of cephalosporin 7 α -hydroxylase. The gels were stained with Coomassie Brilliant Blue R-250.

Characterization of the enzyme

Enzyme preparations possessing specific activities of about 20000 units/mg were used to examine the pH-dependence and temperature-dependence, and to determine *K_m* and *V_{max}*. The decrease in the concentration of the substrate, cephalosporin C (I), was determined by h.p.l.c., with authentic cephalosporin C as a standard. The purest enzyme preparation from the final purification step was used to study the ability to catalyse ring-expansion of penicillin N to deacetoxycephalosporin C and hydroxylation of deacetoxycephalosporin C to deacetylcephalosporin C. Crude cell-free extract was used in control reactions.

RESULTS

Cofactor requirements for cephalosporin hydroxylation

In view of the requirements for 2-oxoglutarate, Fe²⁺ and a reducing agent (L-ascorbic acid) for the methoxylation of cephalosporins by *S. clavuligerus* cell-free extracts, O'Sullivan & Abraham (1980) concluded that the two-step process includes an enzyme having the properties of a dioxygenase. We achieved hydroxylation of cephalosporin C by omission of *S*-adenosylmethionine from cell-free reaction mixtures, and confirmed that the three cofactors are required for efficient hydroxylation, as expected for the dioxygenase nature of cephalosporin 7 α -hydroxylase. In a reaction mixture in which the conversion of cephalosporin C into 7 α -hydroxycephalosporin C was virtually complete (Xiao *et al.*, 1991), the addition of *S*-adenosylmethionine and fresh cell-free extract allowed conversion into 7 α -methoxycephalosporin C with no additional cofactor-dependence.

Purification of cephalosporin 7 α -hydroxylase

Table 1 summarizes the results from each purification step. The ion-exchange step increased the specific activity of cephalosporin 7 α -hydroxylase by over 6-fold with more than 40% of the activity recovered. The (NH₄)₂SO₄ fractionation achieved a slight purification. The enzyme was purified 4-fold by gel-filtration chromatography. The last step, dye chromatography, with Mg²⁺ promoting enzyme binding (Hughes *et al.*, 1982), increased the specific activity of cephalosporin 7 α -hydroxylase to over 20000 units/mg, which was almost 400 times higher than that of the crude cell-free extract. Fig. 1 shows the course of this last step.

SDS/PAGE and *M_r* of cephalosporin 7 α -hydroxylase

Fig. 2 shows the SDS/PAGE results. It can be seen that one band became more prominent as purification proceeded and became the major one after gel filtration, indicating that it was cephalosporin 7 α -hydroxylase. This band was the major one present after dye chromatography. The *M_r* of the SDS-denatured enzyme protein was estimated to be 32000.

Effects of pH and temperature on enzyme activity

Fig. 3 indicates that the optimum pH is in the range 7.3–7.7. The temperature-dependence plot shows highest activity under our conditions in the range 20–30 °C (Fig. 4).

Kinetic studies

Initial cephalosporin C concentrations were varied from 0.1 to 1.0 mM, and the reactions were monitored by h.p.l.c. The initial conversion rates were used for a Lineweaver–Burk analysis (Fig. 5). The results indicate that the *K_m* for cephalosporin C is

Table 1. Purification of *S. clavuligerus* cephalosporin 7 α -hydroxylase

For experimental details see the text.

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Recovery (%)
Crude cell-free extract	1984	117840	59.4	1	100.0
Ion-exchange eluate	152.5	55780	365	6.2	47.3
(NH ₄) ₂ SO ₄ (35–60% saturation) precipitate	91.8	55750	607	10.2	47.3
Gel-filtration eluate	8.4	20507	2441	41.1	17.4
Affi-Gel Blue* eluate	0.1	2343	23430	394.4	2.0

* 20 mM-Mg²⁺ was used to promote enzyme binding. The eluent was 20 mM-Mops buffer, pH 7.5.

Fig. 1. Chromatography on an Affi-Gel Blue column with Mg^{2+} promoting binding of cephalosporin 7 α -hydroxylase

□, Protein content; ◆, cephalosporin 7 α -hydroxylase specific activity.

Fig. 2. SDS/PAGE of fractions from different steps of the purification of cephalosporin 7 α -hydroxylase

Lanes 1 and 7, M_r standards; lane 2, crude cell-free extract; lane 3, eluate from ion-exchanger; lane 4, 35–60% saturation- $(NH_4)_2SO_4$ fraction; lane 5, eluate from gel filtration; lane 6, eluate from Affi-Gel Blue. The cephalosporin 7 α -hydroxylase band is indicated by an arrow. Amount of protein added to lanes 2, 3 and 4 was 1 μ g, that to lane 5 was 0.7 μ g, and that to lane 6 was 0.25 μ g.

0.72 mM and that the V_{max} is 15.4 μ mol of cephalosporin C hydroxylated/min per mg

Absence of deacetoxycephalosporin C synthase and deacetoxycephalosporin C hydroxylase activities from purified cephalosporin 7 α -hydroxylase

In our reaction system containing the three cofactors, the crude cell-free extract catalysed the ring-expansion of penicillin N to deacetoxycephalosporin C (determined by h.p.l.c.) and the hydroxylation of deacetoxycephalosporin C to deacetylcephalosporin C, but the purified enzyme preparation did not. When the reactions with the purified enzyme preparation were extended from 15 min to 80 min, still no deacetoxycephalosporin C or deacetylcephalosporin C formed. These results indicate that the deacetoxycephalosporin C synthase and deacetoxycephalosporin C hydroxylase were removed during the purification, and that cephalosporin 7 α -hydroxylase is a distinct dioxygenase.

Fig. 3. Effect of pH on cephalosporin 7 α -hydroxylase activity

Fig. 4. Effect of temperature on cephalosporin 7 α -hydroxylase activity

Fig. 5. Lineweaver-Burk analysis of cephalosporin 7 α -hydroxylase

DISCUSSION

The deacetoxycephalosporin C synthase and deacetoxycephalosporin C hydroxylase are activities of a bifunctional protein in *Cephalosporium acremonium* (Scheidegger *et al.*, 1984; Samson *et al.*, 1987), but they are distinct proteins in *S. clavuligerus* (Jensen *et al.*, 1985; Cortes *et al.*, 1987). The purified cephalosporin 7 α -hydroxylase resembles deacetoxycephalosporin C synthase and deacetoxycephalosporin C hydroxylase in its molecular size and cofactor requirements, but does not possess either of these activities. Although the complementary experiment has not been performed with deacetoxycephalosporin C synthase and deacetoxycephalosporin C hydroxylase of *S. clavuligerus*, the crude cell-free extract from *C. acremonium*, which contains these enzyme activities, does not show cephalosporin 7 α -hydroxylase activity in our reaction system (results not shown). This is

consistent with the absence of 7 α -hydroxy derivatives of cephalosporins from broths of *C. acremonium*.

We thank Merck and Co. for a fellowship granted to X. X. and acknowledge the interest of K. Bostian and S. W. Drew. We also thank D. Liberman for advice and assistance. The work at Simon Fraser University was funded by the Natural Sciences and Engineering Research Council of Canada.

REFERENCES

- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Cortes, J., Martin, J. F., Castro, J. M., Laiz, L. & Liras, P. (1987) *J. Gen. Microbiol.* **133**, 3165–3174
- Hood, J. D., Elson, A., Gilpin, M. L. & Brown, A. G. (1983) *J. Chem. Soc. Chem. Commun.* 1187–1188
- Hughes, P., Lowe, C. R. & Sherwood, R. F. (1982) *Biochim. Biophys. Acta* **700**, 90–100
- Jensen, S. E., Westlake, D. W. S. & Wolfe, S. (1985) *J. Antibiot.* **38**, 263–265
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- O'Sullivan, J. & Abraham, E. P. (1980) *Biochem. J.* **186**, 613–616
- Piret, J., Resendiz, B., Mahro, B., Zhang, J., Serpe, E., Romero, J., Connors, N. & Demain, A. L. (1990) *Appl. Microbiol. Biotechnol.* **32**, 560–567
- Samson, S. M., Dotzlaf, J. E., Slisz, M. L., Becker, G. W., Frank, R. M. V., Veal, L. E., Yeh, W., Miller, J. R., Queener, S. W. & Ingolia, T. D. (1987) *Bio/Technology* **5**, 1207–1214
- Scheidegger, A., Kuenzi, M. T. & Nuesch, J. (1984) *J. Antibiot.* **37**, 522–531
- Xiao, X., Bowers, R. L., Shin, H., Wolfe, S. & Demain, A. L. (1991) *Appl. Microbiol. Biotechnol.* **35**, 793–797

Received 12 March 1991/31 May 1991; accepted 12 June 1991