

Copurification and Characterization of Deacetoxycephalosporin C Synthetase/Hydroxylase from *Cephalosporium acremonium*

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Deacetoxycephalosporin C synthetase (expandase), which catalyzes ring expansion of penicillin N to deacetoxycephalosporin C (DAOC), has been stabilized in vitro and purified to near homogeneity from the industrially important fungus *Cephalosporium acremonium*. Throughout the purification, the expandase activity remained physically associated with and in a constant ratio of 7:1 to DAOC hydroxylase activity. The latter activity mediates hydroxylation of DAOC to deacetylcephalosporin C (DAC). The copurified expandase/hydroxylase appeared to be monomeric, with a molecular weight of $41,000 \pm 2,000$ and an isoelectric point of 6.3 ± 0.3 . Both catalytic activities required α -ketoglutarate, Fe^{2+} , and O_2 and were stimulated by ascorbate, dithiothreitol, and ATP. The Fe^{2+} requirement was specific, and sulfhydryl groups in the purified protein were apparently essential for both ring expansion and hydroxylation. The kinetics and stoichiometry of DAOC/DAC formation from the expandase/hydroxylase-catalyzed reactions suggested that ring expansion of penicillin N preceded hydroxylation of DAOC.

The remarkable effectiveness of cephalosporins as bactericidal agents in clinical medicine has dictated worldwide use of this class of β -lactam antibiotics. Newton and Abraham isolated and characterized the first cephalosporin, cephalosporin C, as a minor metabolite from the culture broth of *Cephalosporium acremonium* (21). Today, improved strains of that fungus are used to produce large quantities of cephalosporin C for manufacturing many clinically significant parenteral cephalosporin antibiotics. The basic cephalosporin structure allows these antibiotics to survive destruction by most penicillinases produced by penicillin-resistant strains of *Staphylococcus aureus*. Penicillins contain a β -lactam ring fused to a 5-membered thiazolidine ring, and cephalosporins contain a β -lactam ring fused to a 6-membered dihydrothiazine ring. Because of the basic effectiveness of cephalosporins against penicillinase-resistant *S. aureus* pathogens, the enzymatic ring expansion of penicillin into cephalosporin that occurs in cells of *C. acremonium* is of great practical importance.

Significant progress has been made in understanding the biosynthesis of cephalosporins (2). The enzymological basis of ring expansion of penicillins to cephalosporins was demonstrated by Kohsaka and Demain in 1976 (13) with cell extracts of *C. acremonium*. The enzyme, deacetoxycephalosporin C synthetase (commonly called expandase), converts penicillin N to deacetoxycephalosporin C (DAOC) (Fig. 1). Penicillin N accumulation, decreased cephalosporin production, and repression of the expandase have been observed after addition of glucose or NH_4^+ to cultures of *C. acremonium* (8, 28, 30) and after additions of certain nitrogen-containing compounds to *Streptomyces lactamdurans* cultures (7). These data have been interpreted as suggesting that ring expansion represents the rate-limiting step of cephalosporin synthesis.

Isopenicillin N synthetase, the enzyme that catalyzes the cyclization of a tripeptide to an isomeric precursor of penicillin N, has been purified to near homogeneity from *C. acremonium* (9, 22) and *Penicillium chrysogenum* (24). The corresponding genes have recently been cloned, sequenced,

and expressed in *Escherichia coli* (6a, 26). On the other hand, lability of the expandase has prevented significant purification of the enzyme (14, 16, 27). Recently, the expandase and DAOC hydroxylase in extracts of the prokaryote *Streptomyces clavuligerus* were separated (11). DAOC hydroxylase catalyzes hydroxylation of DAOC to deacetylcephalosporin C (DAC) (Fig. 1). Hydroxylation of DAOC to DAC represents the penultimate step in biosynthesis of cephalosporin C. Interestingly, the expandase and hydroxylase activities remained associated when the expandase was purified severalfold from cell extracts of *C. acremonium* (27). Only partially purified preparations of the expandase have been obtained from *C. acremonium* (14, 27) and *S. clavuligerus* (10, 11) owing to the lability of the ring expansion activities. Likewise, there has been no report of highly purified DAOC hydroxylase.

Here we describe conditions for stabilizing the expandase from *C. acremonium*. A novel process using these conditions was devised and used to purify to near homogeneity a protein that catalyzes both ring expansion of penicillin N to DAOC and hydroxylation of DAOC to DAC.

MATERIALS AND METHODS

Growth of the organism. A high-cephalosporin-C-producing strain of *C. acremonium* was grown in a complex liquid medium (23) in 500-ml Erlenmeyer flasks for 96 h. Cells were harvested by centrifugation at $20,000 \times g$ for 10 min, and washed with 50 mM Tris hydrochloride, pH 7.5, in the presence of 1.0 M KCl and again with the buffer without KCl prior to preparation of a crude extract.

Enzyme assays. The expandase and hydroxylase activities were determined by high-performance liquid chromatography (HPLC) assays modified from those described previously (8, 18, 27). The expandase-catalyzed reaction, unless otherwise specified, was conducted for 15 min at 30°C with 0.28 mM penicillin N, 0.30 mM α -ketoglutarate (α -KG), 0.06 mM $FeSO_4$, 0.25 mM ascorbate, 1.00 mM dithiothreitol (DTT), 0.05 mM ATP, and 0.0003 to 0.003 U of the enzyme in 1 ml of 50 mM Tris hydrochloride, pH 7.5. The hydroxylase-catalyzed reaction, except as indicated, was conducted similarly at 36°C with 0.028 mM DAOC instead of penicillin

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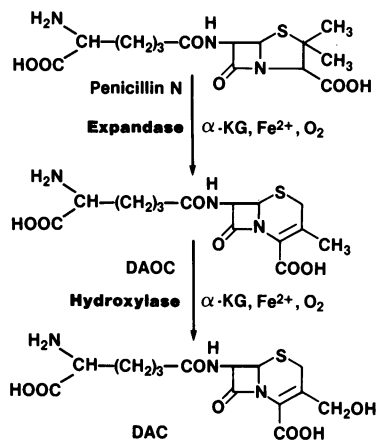


FIG. 1. Expandase- and hydroxylase-catalyzed reactions.

N as one substrate. The enzymatic reactions were interrupted by addition of 1 ml of ethanol, and supernatant solutions which contained reaction products were obtained by centrifugation at $4,000 \times g$ for 5 min. The expandase activity was determined by monitoring formation of both DAOC and DAC from penicillin N because of the putative bifunctionality of the enzyme (27). The hydroxylase activity was determined by monitoring formation of DAC from DAOC.

DAOC and DAC from 20- to 100- μl samples of the supernatant solutions were quantitated by an HPLC procedure with external standards. HPLC components were model 721 system controller, model 730 data module, model 510 EF pumps, model 710B Waters intelligent sample processor, and Lambda-Max model 481 LC spectrophotometer (Waters Associates, Milford, Mass.). DAC and DAOC were separated by a radially compressed $\mu\text{Bondapak-NH}_2$ column (0.8 by 10 cm) (Waters) with a mobile phase of 2% acetate-0 to 4% methanol-6 to 7% acetonitrile-87 to 92% water, pH 3.8, a flow rate of 1.5 to 2.0 ml/min, and detection at 260 nm. The HPLC assays were reproducible with 2% deviations for duplicate analyses of both the expandase- and the hydroxylase-catalyzed reactions. One unit of expandase activity was defined as the amount of enzyme required to cause the formation of 1 μmol of DAOC plus DAC per min from penicillin N. One unit of hydroxylase activity was defined as the amount of enzyme required to cause the formation of 1 μmol of DAC per min from DAOC.

The protein content was determined by the Bradford method (6) with bovine serum albumin fraction V as the standard.

Expandase stabilization. The instability of the expandase from *C. acremonium* appeared to hamper previous enzyme purification attempts (14, 27). The expandase stability at -20°C was described recently (16); e.g., DTT was shown to effectively reactivate the enzyme after its inactivation. The expandase stabilization was further developed to facilitate enzyme purification. At 4°C , the expandase from a crude extract of *C. acremonium* was stable for a week in the presence of 2 mM phenylmethylsulfonyl fluoride (PMSF), 10% ethanol, 1 mM DTT, and 1 mM ascorbate. Partially purified enzyme preparations from DEAE-cellulose column eluates did not remain stable under the above conditions; however, some protection was observed with glycerol, sucrose, or penicillin N. DAOC and $\alpha\text{-KG}$ had no effect on

enzyme stabilization. A highly purified expandase prepared by DEAE-cellulose chromatography, Sephacryl S-200 gel filtration, and hydroxylapatite chromatography was about 90% stable for a week in the presence of 10% glycerol, 10% ethanol, 10 mM DTT, and 10 mM ascorbate. Removal of DTT and ethanol from the hydroxylapatite eluate resulted in complete inactivation of the expandase in 1 and 4 days, respectively, whereas deletion of either glycerol or ascorbate led to enzyme inactivation in 7 days. Also, initial purification studies implicated ammonium sulfate in partial destabilization of the expandase and therefore it was not used during the enzyme purification.

Expandase purification. Enzyme purification was performed between 0 and 4°C , and all buffers were thoroughly degassed prior to use. Fresh cells (wet weight, 600 g) were suspended in 50 mM Tris hydrochloride, pH 7.5, in the presence of 10% glycerol, 10% ethanol, 10 mM DTT, and 10 mM ascorbate (GEDA buffer) to a total volume of 1 liter and broken by sonic treatment at 4°C or below. Multiple PMSF additions were made to a final concentration of 2 mM during the sonication. DNase and MgSO_4 were added at 1 $\mu\text{g}/\text{ml}$ and 10 mM, respectively. The supernatant fraction, which was obtained by centrifugation at $40,000 \times g$ for 30 min, was used as the crude extract (see Table 1, step 1) without further treatment. The crude extract was loaded onto a DEAE-Trisacryl LS column (5 by 30 cm) previously equilibrated with GEDA buffer. The expandase was not retained by DEAE-Trisacryl in 50 mM Tris hydrochloride buffer, pH 7.5 (though it was retained in 15 mM Tris hydrochloride buffer, pH 7.5); however, retention of contaminating proteins led to some (i.e., 1.6-fold) purification of the enzyme in the filtrate (Table 1, step 2). The filtrate was loaded onto a DEAE-cellulose column (2.5 by 41 cm) previously equilibrated with GEDA buffer. The column was washed with 4 column volumes of the buffer in the presence of 0.05 M KCl, after which a linear gradient of 0.05 to 0.60 M KCl in GEDA buffer in a total volume of 800 ml was applied. Fractions of 10 ml were collected at a flow rate of 25 ml/h. The expandase was eluted between 0.04 and 0.06 M KCl as one major and two minor activity peaks; about 75% of the total activity resided within the major peak. The fractions from the major peak with specific activities greater than 0.088 U/mg were pooled (Table 1, step 3), concentrated to 9.5 ml by ultrafiltration with an Amicon PM30 membrane, and loaded onto a Sephacryl S-200 column (5 by 85 cm) previously equilibrated with GEDA buffer. Fractions of 10 ml were collected at a flow rate of 40 ml/h. The fractions with specific activities of at least 0.33 U/mg were combined (Table 1, step 4) and loaded onto a hydroxylapatite column (1.6 by 95 cm) previously equilibrated with GEDA buffer in the presence of 20 mM potassium phosphate. The column was washed with 2 column volumes of the same buffer. Elution of the expandase was made by a stepwise gradient with 100 ml of GEDA buffer containing 30, 40, 60, 80, and 100 mM potassium phosphate. Fractions of 5 ml were collected at a flow rate of 15 ml/h. The expandase was eluted as one major and several minor activity peaks; the major peak (Table 1, step 5, for fractions with specific activities greater than 0.558 U/mg) contained about 80% of the total activity. PMSF was added at 0.25 mM to individual fractions containing the expandase activity. The fraction from the major peak with the highest specific activity (0.827 U/mg) was further purified by fast protein liquid chromatography (FPLC; Pharmacia) with Mono Q. A portion (5.6 mg of protein) of the fraction was loaded onto a Mono Q column (0.5 by 5 cm) previously equilibrated with GEDA buffer, and the enzyme was eluted

with a linear gradient of 0 to 0.4 M KCl in the buffer in a total volume of 32 ml. Fractions of 1 ml were collected at 30 ml/h. The activity and protein elution patterns from Mono Q FPLC are shown in Fig. 2A. The other fractions from hydroxylapatite chromatography with specific activities greater than 0.558 U/mg, i.e., from the main peak, were combined and loaded onto a DEAE-Sepharose column (1.6 by 95 cm) previously equilibrated with GEDA buffer. The column was washed with 2 column volumes of the buffer in the presence of 0.05 M KCl, and a linear gradient of 0.05 to 0.60 M KCl in the buffer in a total volume of 400 ml was applied. Fractions of 5 ml were collected at a flow rate of 15 ml/h. The activity and protein elution patterns from DEAE-Sepharose chromatography are shown in Fig. 2B.

Molecular weight determination. The molecular weight of active expandase from the DEAE-cellulose eluate (Table 1, step 2) was estimated by gel filtration on a Bio-Gel A0.5m column (1.6 by 100 cm), which had been equilibrated with 50 mM Tris hydrochloride, pH 7.5, in the presence of 1 mM DTT and 1 mM ascorbate and calibrated with yeast alcohol dehydrogenase (80,000), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), and RNase (13,700). The minimal molecular weight of the enzyme from the Mono Q eluate (Fig. 2A) was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with protein molecular weight standards from Bio-Rad Laboratories.

SDS-PAGE. SDS-PAGE for denatured proteins was performed as described by Laemmli (15). Precast gradient polyacrylamide gels (Separagels; Integrated Separation Systems, Newton, Mass.) were routinely used. PAGE for native proteins was conducted by the method of Blackshear (5). After electrophoresis, proteins in the separating gels were visualized by staining with Coomassie brilliant blue R-250.

Amino acid analysis. The DEAE-Sepharose eluate (Fig. 2B), after further purification by Mono Q FPLC (Fig. 3C), was hydrolyzed in 6 N HCl at 110°C for 24, 48, 72, or 96 h. Amino acids were analyzed by a Beckman amino acid analyzer (model 6300) with a computerized integration system. Threonine and serine were extrapolated to time zero of hydrolysis. Cysteine was estimated as cysteic acid after dimethyl sulfoxide treatment (29). Tryptophan was determined by hydrolysis with thioglycolic acid (17).

Isoelectric focusing. The isoelectric point of the expandase was determined as described previously (3, 4). Electrophoresis was conducted with 5% (pH 3.5 to 10) ampholytes (Pharmacia) in a 4% acrylamide gel. The proteins were visualized with silver stain.

Chemicals. HPLC-grade acetonitrile and methanol were purchased from Mallinckrodt, Inc. Paris, Ky., and HPLC-grade acetic acid was obtained from J. T. Baker Chemical Co., Phillipsburg, N.J. Highly purified penicillin N, DAOC, DAC, and analogs of penicillin N were available from Eli Lilly and Co., Indianapolis. DTT, Tris base, α -KG, ascorbate, PMSF, 1,10-phenanthroline, EDTA, *p*-hydroxymercuribenzoate (*p*-HMB), *N*-ethylmaleimide (NEM), and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) were obtained from Sigma Chemical Co., St. Louis, Mo. Other chemicals were analytical grade. DEAE-cellulose (DE52) was purchased from Whatman, Inc., Clifton, N.J. DEAE-Trisacryl LS was obtained from LKB Instruments, Inc., Gaithersburg, Md. Hydroxylapatite (Hyapatite C) was purchased from Clarkson Chemical Company, Inc., Williamsport, Pa. Sephacryl S-200 and DEAE-Sepharose were obtained from Pharmacia, Inc., Piscataway, N.J.

TABLE 1. Expandase purification

Step	Total protein (mg)	Total activity		Sp act (U/mg)	Purification (fold)
		U	Yield (%)		
Crude extract	12,500	485	100	0.039	1.0
DEAE-Trisacryl filtrate	6,200	393	81	0.063	1.6
DEAE-cellulose eluate	900	138	29	0.154	3.9
Sephacryl S-200 eluate	260	119	25	0.453	11.7
Hydroxylapatite eluate	90	57	12	0.633	16.2

RESULTS

Expandase identification and purity. The chromatographic procedure for purification of active expandase is summarized in Table 1. Further purification by Mono Q FPLC (Fig. 2A) or DEAE-Sepharose chromatography (Fig. 2B) led to severe inactivation of the expandase. However, SDS-PAGE revealed selective enrichment of a 41,000-dalton protein (Fig. 3A), which was the main protein after Mono Q FPLC (Fig. 3B) or DEAE-Sepharose chromatography (Fig. 3C). On the basis of densitometric scanning, the main protein from the peak activity fraction of Mono Q FPLC (Fig. 2A) accounted for 95% of the total protein (Fig. 3B). The amounts of two detectable minor proteins present in fractions 15 to 19 (Fig. 3B) were inversely related to the expandase activity (Fig. 2A) and were not apparently enriched throughout the purification (Fig. 3A). The chromatographic and electrophoretic purification data indicate that the main protein is the expandase. A near electrophoretically

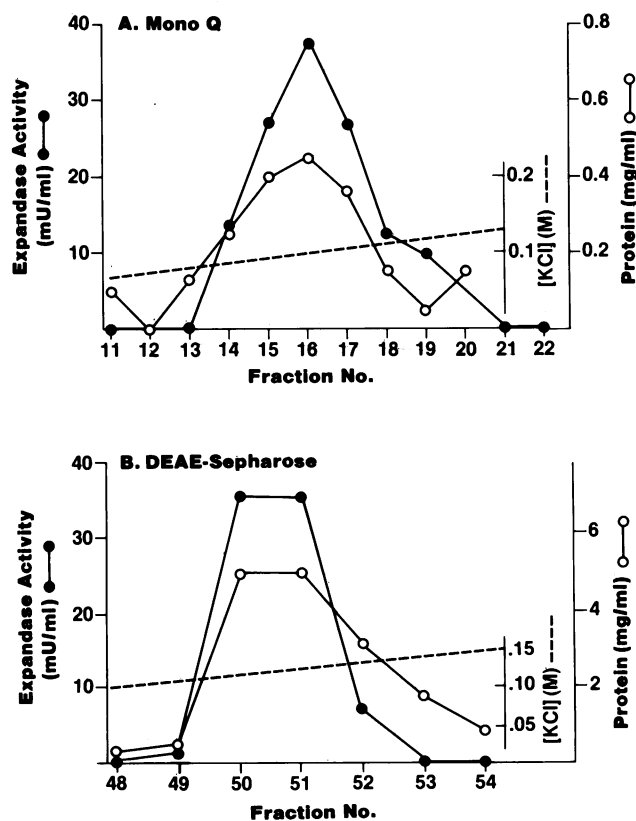


FIG. 2. Mono Q FPLC (A) and DEAE-Sepharose chromatography (B) of expandase.

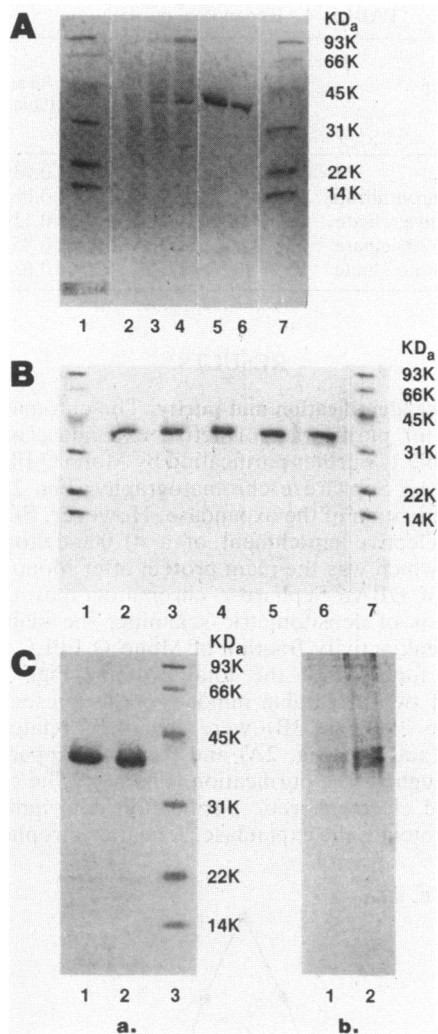


FIG. 3. (A) Monitoring expandase purification by SDS-PAGE with 10 to 20% gradient gel. Lanes contained protein standards (2.5 μ g) (lanes 1 and 7) or 10 μ g of crude extract (lane 2), DEAE-Trisacryl filtrate (lane 3), DEAE-cellulose eluate (lane 4), hydroxylapatite eluate (lane 5), or Mono Q eluate (lane 6). (B) SDS-PAGE for Mono Q FPLC of expandase with 10 to 20% gradient gel. Lanes contained protein standards (2.5 μ g) (lanes 1 and 7) or 10 μ g of Mono Q fraction 15 (lane 2), fraction 16 (lane 3), fraction 17 (lane 4), fraction 18 (lane 5), or fraction 19 (lane 6). (C) SDS-PAGE (a) and PAGE (b) for DEAE-Sepharose chromatography or further Mono Q FPLC of expandase with 12% gel. (a) Lanes contained protein standards (2.5 μ g) (lane 3) or 10 μ g of DEAE-Sepharose eluate (lane 1) or further Mono Q eluate (lane 2). (b) Lanes contained 10 (lane 1) and 20 (lane 2) μ g of further Mono Q eluate. Sizes are shown in kilodaltons.

homogeneous but inactive expandase (Fig. 3C) was obtained by Mono Q FPLC of the DEAE-Sepharose eluate (Fig. 2B).

Copurification of expandase/hydroxylase. The expandase and hydroxylase activities were not separable by DEAE-Trisacryl chromatography, Sephacryl S-200 gel filtration, hydroxylapatite chromatography, or Mono Q FPLC (Fig. 4). In spite of major activity loss of the expandase during the last anion-exchange chromatography, the ratio of the hydroxylase and expandase activities remained constant at 0.15 ± 0.04 during the entire chromatographic purification.

Physical properties. The molecular weight of active

expandase/hydroxylase, as estimated by gel filtration with Bio-Gel A0.5m, was 43,000. The minimal molecular weight of the purified protein, as determined by SDS-PAGE, was 41,000. The isoelectric point of the protein was estimated as 6.3 ± 0.3 . Thus, the expandase/hydroxylase behaved like a weakly acidic monomer during its purification at pH 7.5.

Optimal catalysis. Some reaction parameters for optimal catalysis by the expandase/hydroxylase are listed in Table 2. The expandase and the hydroxylase exhibited somewhat different pH and temperature optima. At the optimal pH values, substitution of Tris hydrochloride by potassium phosphate caused 40 and 20% reductions in the expandase and hydroxylase activities, respectively. No optimal ionic strength of Tris hydrochloride was observed for the expandase, i.e., except for a slight upward interruption at 75 mM, expandase activity decreased with increasing concentration of the buffer in the range from 5 to 500 mM. Hydroxylase activity was maximal between 5 and 75 mM and dropped to 70% of the maximal level at 200 mM. External α -KG and Fe^{2+} were required for expression of the expandase and hydroxylase activities. In addition, both enzymes were stimulated by DTT, ascorbate, and ATP. Both reducing agents were needed for maximal activity of the expandase; however, DTT stimulation of the hydroxylase was observed between 0 and 200 mM ascorbate only. ATP was moderately stimulatory to the expandase and slightly but detectably stimulatory to the hydroxylase. Less stimulation for the expandase and some inhibition for the hydroxylase were observed at high ATP concentrations; e.g., 20% stimulation and 25% inhibition at 0.8 mM ATP for the expandase and the hydroxylase, respectively. The K_m of the expandase for penicillin N and that of the hydroxylase for DAOC was similar, and those of the two enzymes for α -KG were identical (Table 2).

Stoichiometry of expandase/hydroxylase-catalyzed reactions. The product/substrate ratio during a 25-min expandase/hydroxylase-catalyzed conversion of penicillin N to DAOC and DAC was maintained at 1:1 (Fig. 5A). DAOC was formed prior to DAC. Similarly, DAOC was quantitatively converted to DAC at 1:1 during a 60-min hydroxylase-catalyzed reaction (Fig. 5B). The conversion of either substrate was complete under the reaction conditions.

Substrate specificity and inhibition of expandase. No ring expansion occurred under the optimal catalytic conditions for the expandase (Table 2) when penicillin N was replaced by isopenicillin N, penicillin G, penicillin V, ampicillin, or 6-aminopenicillanic acid. When examined at a twofold-higher concentration than that of penicillin N (i.e., 280 μ M), none of the side chain analogs showed detectable inhibition to the expandase.

Effect of metal ions. Fe^{2+} was required for both expandase and hydroxylase activities; it could not be replaced by Mg^{2+} , Mn^{2+} , Co^{2+} , Ca^{2+} , Cu^{2+} , Ni^{2+} , Zn^{2+} , Na^+ , or K^+ . Both enzymes were slightly more active with Fe^{2+} than with Fe^{3+} in the presence of DTT or ascorbate. In contrast to the narrow metal ion requirement for Fe^{2+} , the two enzymes were specifically but more broadly inhibited by metal ions. Zn^{2+} was most inhibitory and Co^{2+} was slightly inhibitory to both enzymes. In addition, the expandase was more sensitive than the hydroxylase to inhibition by Mn^{2+} , whereas only the hydroxylase was inhibited slightly by Cu^{2+} .

Effect of metal chelators and sulfhydryl reagents. The expandase and the hydroxylase were similarly sensitive to inhibition by EDTA and 1,10-phenanthroline (Table 3). Both enzymes were similarly susceptible to inhibition by ρ -HMB and DTNB, differentially susceptible to inhibition by NEM,

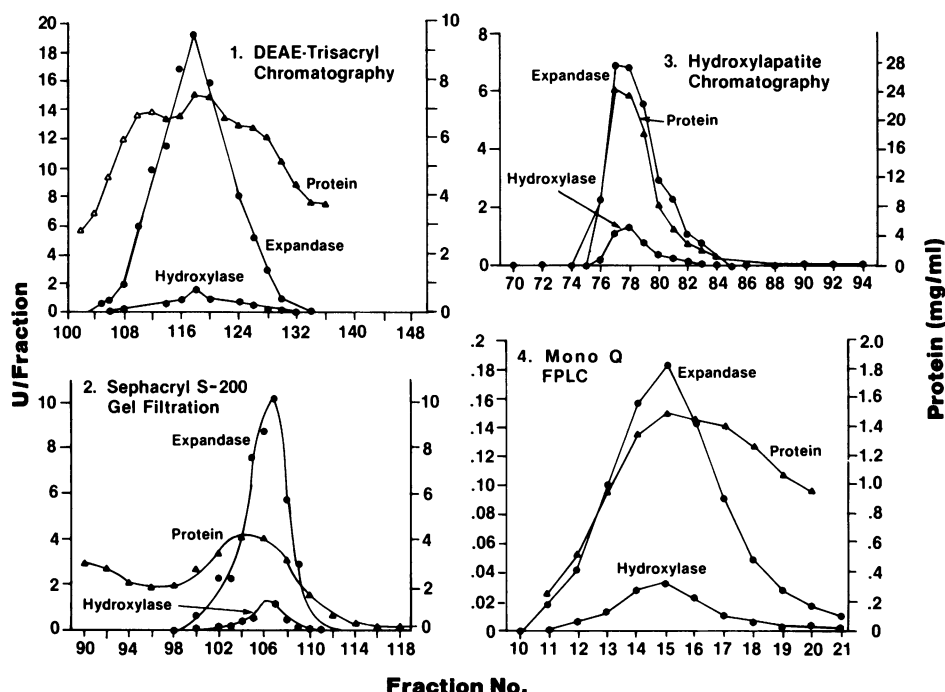


FIG. 4. Copurification of expandase/hydroxylase. Conditions for this similar but separate purification are same as those described in Materials and Methods except that GEDA buffer with 15 mM Tris hydrochloride was used during DEAE-Trisacryl chromatography and the enzymes were eluted from Mono Q by a gradient of 0.015 to 0.5 M Tris hydrochloride. The 41,000-dalton protein of Mono Q fraction 15 accounted for, on the basis of SDS-PAGE, 90% of the total protein.

and not affected by iodoacetic acid (Table 3). After complete inactivation by 0.2 mM DTNB in 1 min at 4°C, addition of 2 mM DTT restored the initial expandase activity fully and 60% of the initial hydroxylase activity in 30 min.

Expandase/hydroxylase stability. After a 1-h preincubation at 4°C, the expandase and the hydroxylase were generally stable within a wide pH range from 5 to 10 (Fig. 6A). With a 10-min preincubation at pH 7.5, the thermal inactivation kinetics of the expandase and the hydroxylase were indistinguishable (Fig. 6B). Also, the inactivation rates of the two enzymes at 45°C were almost identical.

Amino acid composition. As a guide for the initial structural analysis, the amino acid composition of the expandase/hydroxylase is shown in Table 4.

DISCUSSION

We describe an effective procedure for purification of the expandase from *C. acremonium* to near electrophoretic homogeneity. The procedure is based on enzyme stabilization, an effective HPLC assay, and a simple set of conven-

tional liquid chromatography methods. The expandase, which was identified as a 41,000-dalton protein, was the major SDS-PAGE band (i.e., about 80% of the total protein) from the hydroxylapatite eluate (Fig. 3A) and was highly active (Table 1). Further purification by anion-exchange chromatography with either Mono Q (Fig. 2A) or DEAE-Sepharose (Fig. 2B) led to a highly purified (Fig. 3B and C) but severely inactivated expandase. The enzyme inactivation might have been caused by use of KCl in the gradient elution. A recently described general mechanism for the nonenzymatic cleavage of enzymes (12) involves formation of localized reactive oxygen species in the presence of DTT or ascorbate, O₂, and iron or copper. Those conditions, though likely present throughout the expandase purification, did not cause any measurable degradation of the enzyme (Fig. 2 and 3). Therefore, KCl-related expandase inactivation was apparently not accompanied by protein degradation. Substitution of KCl by Tris hydrochloride in the gradient elution of the expandase significantly decreased the enzyme inactivation (about 10-fold); however, resolution of

TABLE 2. Reaction parameters for optimal catalysis of expandase/hydroxylase^a

Enzyme	Optimum pH	Optimum temp (°C)	Minimal saturation (μM)		O ₂ required ^b	Maximal reactivation or stimulation ^c (mU) by:			K _m (μM)			V _{max} ^d
			α-KG	Fe ²⁺		1.0 mM DTT	0.25 mM ascorbate	0.05 mM ATP	Penicillin	DAOC	α-KG	
Expandase	7.5–7.8	26–34	200	60	Yes	0→5.0	0→9.0	3.8→5.2	29	— ^e	22	0.827
Hydroxylase	7.3	36–38	200	60	Yes	0→0.023	0→0.081	0.057→0.061	—	20	22	0.127

^a Determined from the hydroxylapatite eluate (see Table 1).

^b Determined under N₂ and from reference 11.

^c From base → maximal rate.

^d Micromoles of product formed per minute per milligram of protein, estimated from the fraction with the highest specific activity (see text).

^e —, Not a substrate.

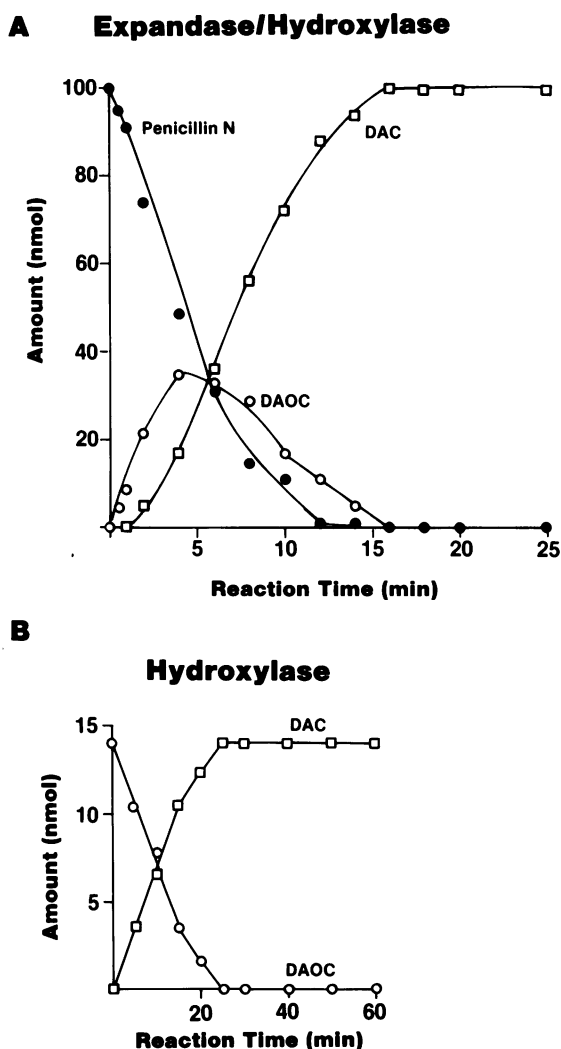


FIG. 5. Stoichiometric analysis of expandase/hydroxylase-catalyzed conversion of penicillin N to DAOC and DAC (A) and hydroxylase-catalyzed conversion of DAOC to DAC (B). Quantitation of penicillin N (A) was corrected for its degradation at 1.5%/min.

the enzyme from contaminating proteins also decreased. On the basis of its wide pH stability (Fig. 6A), the expandase might be further purified with a pH gradient (e.g., by chromatofocusing) during a final chromatographic step.

From coelution of the expandase and the hydroxylase by DEAE-Trisacryl chromatography and their comigration by isoelectric focusing, Scheidegger et al. (27) suggested that the expandase/hydroxylase was a bifunctional enzyme. The putative bifunctionality is substantiated by our purification and characterization studies demonstrating (i) chromatographic and electrophoretic inseparability (Fig. 3 and 4), (ii) common requirements for α -KG, Fe^{2+} , and O_2 and stimulation by DTT, ascorbate, and ATP (Table 2), (iii) inhibition by metal chelators and sulfhydryl agents, including Zn^{2+} (Table 3), (iv) similar pH and temperature stability patterns (Fig. 6), and (v) very similar K_m values for the three substrates (Table 2). Quantitative but not qualitative functional differences for the two enzymes, however, were observed in sensitivity to Mn^{2+} and NEM (Table 3), stimulation by DTT and ATP (Table 2), and reversibility of DTNB inhibition. Thus, al-

though the physical and functional properties strongly suggest bifunctionality of the expandase/hydroxylase, the possibility for copurification of two highly similar enzymes has not been eliminated. The functional variation could be a reflection of two different active sites from a bifunctional expandase/hydroxylase or of two very similar enzymes. One feasible approach for conclusively demonstrating bifunctionality, which is being pursued, is to clone the structural gene of the 41,000-dalton protein into *E. coli* with an oligonucleotide probe constructed according to a sequence of the protein and to demonstrate simultaneous expression of the expandase and hydroxylase activities. In contrast to copurification of the expandase/hydroxylase from *C. acremonium*, the expandase and the hydroxylase from *S. clavuligerus* could be separated by an initial anion-exchange chromatography step (11). Comparative structural and functional properties of the two enzymes from the eucaryotic and procaryotic cephalosporin C producers are needed to elucidate the basis of this interesting and probably evolutionary variation.

The expandase and the hydroxylase have a common set of complex catalytic properties. Except for their respective β -lactam substrates, both enzymes required α -KG, Fe^{2+} , and O_2 and were stimulated by ascorbate, DTT, and ATP. The common requirements and stimulations were shown for partially purified (14, 16, 27) as well as highly purified (Table 2) expandase/hydroxylase. The participation of α -KG, Fe^{2+} , O_2 , and ascorbate in their catalytic reactions suggests that both the expandase and the hydroxylase are α -KG-coupled dioxygenases (1). Since no net oxygenation occurs in the ring expansion, a transient oxygen addition is presumably followed by a deoxygenation step (20, 25) in the expandase-catalyzed reaction. The tight stoichiometric conversion (1:1) of penicillin N to DAOC plus DAC by the expandase/hydroxylase (Fig. 5A) and that of DAOC to DAC by the hydroxylase (Fig. 5B) indicate that no alternative metabolic route other than penicillin N \rightarrow DAOC \rightarrow DAC was operative under the reaction conditions of the two enzymes. The former stoichiometry also suggests that oxygenation of penicillin N rather than subsequent deoxygenation of an oxygenated intermediate is the rate-limiting step of the expandase-catalyzed ring expansion reaction. Also, the existence of possible enzyme-bound penicillin intermediates cannot be ruled out on the basis of the stoichiometric data.

TABLE 3. Effect of metal chelators and sulfhydryl reagents on expandase/hydroxylase^a

Addition	Concn (mM)	Relative activity (%)	
		Expandase	Hydroxylase
None		100	100
O-Phenanthroline	0.06	78	83
	0.60	0	0
EDTA	0.06	67	95
	0.60	11	17
ρ -HMB	0.10	11	12
	1.00	0	0
DTNB	0.10	53	43
	1.00	0	0
NEM	0.10	87	86
	1.00	49	3
Iodoacetic acid	0.10	92	98
	1.00	96	98

^a Enzymatic reactions were conducted with the hydroxylapatite eluate (Table 1) in the presence of 0.06 mM FeSO_4 , 0.10 mM DTT, and 0.67 mM ascorbate. Preincubation of the enzyme and an inhibitor was done at 4°C for 1 min.

Functional Fe^{2+} and sulfhydryl groups have been implicated for the expandase/hydroxylase. The specific requirement of Fe^{2+} for expression of the two activities indicates that iron is bound loosely to and dissociates readily from the expandase/hydroxylase in vivo or during enzyme purification. The functionality of Fe^{2+} was also shown by sensitivity of the expandase/hydroxylase to EDTA and 1,10-phenanthroline (Table 3). Inhibition of both the expandase and the hydroxylase by Zn^{2+} , which is known to bind sulfhydryl groups (19), and by ρ -HMB, DTNB, and NEM (Table 3) suggests that at least one sulfhydryl group is apparently essential for either enzyme.

DTT and ascorbate contribute to the stability and optimal catalysis (Table 2) of the expandase, presumably by keeping iron in the reduced form or by protecting essential sulfhydryl groups of the enzyme (16, 27). Greater expression of both activities with Fe^{2+} than with Fe^{3+} in the presence of DTT or ascorbate suggests that, in optimal catalysis by the expandase/hydroxylase, one common function of DTT and ascorbate is iron reduction. Complete inhibition of the two enzymes by ρ -HMB and DTNB in the presence of ascorbate (Table 3) and reversible reactivation by DTT subsequent to DTNB inactivation indicate that another function of DTT

TABLE 4. Amino acid composition of expandase/hydroxylase

Amino acid	No. of residues per 41,000 daltons
Asx (Asp + Asn)	37
Thr	24
Ser	26
Glx (Glu + Gln)	35
Pro	21
Gly	31
Ala	34
Val	32
Cys	6
Met	5
Ile	8
Leu	27
Tyr	10
Phe	20
His	6
Lys	17
Arg	29
Trp	3

but not ascorbate is for maintenance of apparently essential sulfhydryl groups of the expandase/hydroxylase.

How ATP participates in catalysis by the expandase/hydroxylase is far from clear. ATP was used in the assay for *C. acremonium* expandase (14) but not in that for *S. clavuligerus* expandase (10). In two separate studies, ATP was moderately stimulatory to the expandase and slightly stimulatory to the hydroxylase at the optimal concentrations (Table 3) (27). Whether the ATP stimulation is a direct or indirect functional property of the two enzymes has not been elucidated.

The expandase/hydroxylase from this study is larger (M_r 41,000 versus 31,000 to 33,000), less acidic (pI of 6.3 versus 4.6), and much more active (Tables 1 and 2) than those described previously (14, 27). The origin of the physical and activity variations is not known; however, it may involve strain differences or in vitro proteolytic degradation. In spite of the significant differences, very little variation was observed in the K_m of the expandase/hydroxylase for penicillin N, DAOC, and α -KG. The substrate specificity of the expandase appears to be less restrictive with α -KG (14) than with penicillin N. The lack of ring expansion from and the absence of enzyme inhibition by the analogs of penicillin N examined so far indicate a high selectivity of the expandase.

The availability of a highly purified and active expandase/hydroxylase from *C. acremonium* or *E. coli* (via gene cloning) should facilitate a systematic search into novel ring expansions of scientific and industrial interest. The putative bifunctionality and narrow specificity for penicillin N may make the expandase/hydroxylase a desirable model for the analysis of functional modification by protein engineering.

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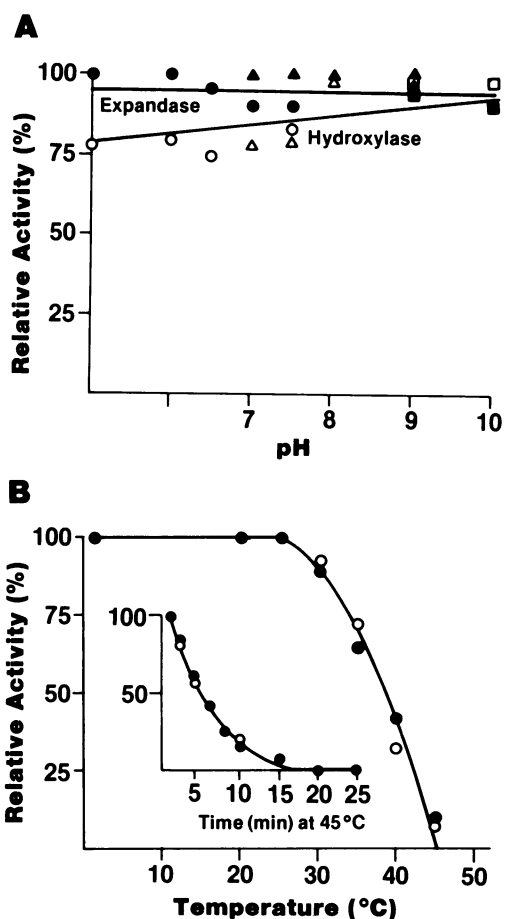


FIG. 6. Stability of expandase/hydroxylase to pH (A) and temperature (B). The pH stability studies were conducted with potassium phosphate, pH 5.0 to 7.5 (●, ○), Tris hydrochloride, pH 7.0 to 9.0 (▲, △), and sodium glycinate, pH 9.0 to 10.0 (■, □). The temperature stability studies were performed between 1 and 45°C and at 45°C for both the expandase (●) and the hydroxylase (○).

ADDENDUM IN PROOF

DAOC synthetase (expandase) was recently purified to near homogeneity from a low-cephalosporin-C-producing strain of *Cephalosporium acremonium* (J. E. Baldwin, R. M. Adlington, J. B. Coates, M. J. C. Crabbe, N. P. Crouch, J. W. Keeping, G. C. Knight, C. J. Schofield, H. H. Ting, C. A. Vallejo, M. Thorniley, and E. P. Abraham, *Biochem. J.*, in press). The expandase could not be separated from DAOC hydroxylase by ion-exchange, dye-ligand or hydrophobic chromatography, which led the authors to suggest that the two enzyme activities are properties of a single 40,000-dalton protein.

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