Macrolide Antibiotic Biosynthesis: Isolation and Properties of Two Forms of 6-Deoxyerythronolide B Hydroxylase from *Saccharopolyspora erythraea* (*Streptomyces erythreus*)†

Ali Shafiee and C. Richard Hutchinson*

School of Pharmacy and Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706

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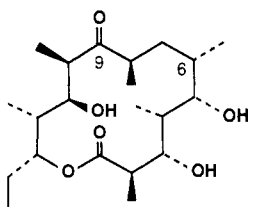
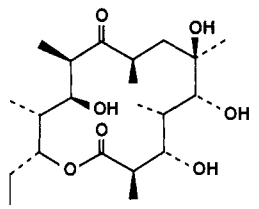
ABSTRACT: A cytochrome P-450 monooxygenase that catalyzes the hydroxylation of 6-deoxyerythronolide B, an intermediate of erythromycin A biosynthesis in *Saccharopolyspora erythraea* (formerly *Streptomyces erythreus*), was resolved into two forms, P-450$_{I}$ and P-450$_{II}$, by hydroxylapatite chromatography. These two proteins were purified to homogeneity from the CA 340 strain and found to have a P-450 content of 17.5 and 15.2 nmol/mg of protein, respectively. Either enzyme catalyzed the NADPH-dependent hydroxylation of 6-deoxyerythronolide B and (9*R*)- or (9*S*)-9-deoxo-9-hydroxy-6-deoxyerythronolide B in vitro when reconstituted with other electron-transport components from *S. erythraea*. Both of them had a M_r of 44 220 ± 1350, a p*I* of 4.6, similar amino acid compositions, and an identical N-terminal sequence for the first five amino acids. They also showed identical antigenicity and cross-reactivity against polyvalent and specific antibodies and contained cytochrome P-450 in the low spin state with absorption maxima at 416, 532, and 565 nm. Their distinguishing characteristics were different activities toward the (9*S*)-9-deoxo-9-hydroxy-6-deoxyerythronolide B substrate and slightly different absorbance maxima in their dithionite-reduced CO-complexed spectra.

Through metabolite isolation, isotope feeding experiments, and studies of blocked mutants, the biosynthesis of erythromycin A, a macrolide antibiotic produced by *Streptomyces erythreus* (McGuire et al., 1952) [reclassified as *Saccharopolyspora erythraea* (Labeda, 1987)], is known to involve three major stages (Corcoran, 1981): formation of the 14-membered macrolactone erythronolide B (EB),[1] formation and stepwise



6D



EB

addition of the two deoxy sugars mycarose and desosamine to this lactone to produce erythromycin D, and then C-12 hydroxylation and C-3″ O-methylation of the latter compound to form erythromycin A. Several enzymes that may provide essential precursors for the formation of 6-deoxyerythronolide B (6D) have been purified from *S. erythraea* [Hunaiti & Kolattukudy, 1982, 1984a,b; reviewed in Seno and Hutchinson (1986)], and crude cell-free systems have been developed that contain the C-12 hydroxylase (Corcoran, 1981) and C-3″ *O*-methyltransferase (Corcoran, 1975) activities.

With an interest in the genetics and biochemistry of erythromycin formation (Weber et al., 1985; Seno & Hutchinson, 1986; Yamamoto et al., 1986), we chose to study the enzyme for the hydroxylation of 6D to EB because a convenient assay was available to guide its purification. The partial purification of 6D hydroxylase by Corcoran and Vygantas (1982) established that it is a cytochrome P-450 enzyme with terminal oxidase activity which functions through an NADPH-dependent electron-transport pathway. Other data (Corcoran, 1981; Petzoldt & Kieslich, 1969) suggest that the level of this enzyme may be inducible in vivo. Such properties are characteristic of cytochrome P-450 enzymes isolated from other prokaryotes (Unger et al., 1986) and eukaryotes (Guengerich, 1979; Aoyama et al., 1984), systems for which much is known about the protein structure (Guengerich et al., 1982; Poulos et al., 1985), activity (Sanglard et al., 1984), inducibility (Fisher et al., 1981; Walz et al., 1982), and biological role (Guengerich, 1979; Trazaskos, 1984). To establish comparable knowledge about a cytochrome P-450 enzyme in the *Streptomyces* and to identify the gene encoding 6D hydroxylase in the *ery* gene cluster (Stanzak et al., 1986), we purified 6D hydroxylase from *S. erythraea* to homogeneity and found that this enzyme apparently exists in two forms in vivo. The two forms have very similar physical and catalytic properties, which raises questions about the reason for their presence in this microorganism and their possible role in the regulation of erythromycin A production.

Experimental Procedures

Materials. PMSF, ammonium sulfate (grade III), benzamidine, *p*-aminobenzamidine, sodium cholate, Lubrol Px, FAD, NADP$^+$, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, spinach ferredoxin–NADP$^+$ reductase, spinach ferredoxin (type III), and anti-rabbit IgG–alkaline phosphatase conjugate were purchased from Sigma, St. Louis, MO; bacterial alkaline phosphatase was from BRL, Gaithersburg, MD;

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[1] Abbreviations: 6D, 6-deoxyerythronolide B; EB, erythronolide B; (9*R*)- and (9*S*)-9-deoxo-9-hydroxy-6D, C-9 epimers of 9-deoxo-9-hydroxy-6-deoxyerythronolide B; (9*R*)- and (9*S*)-9-deoxo-9-hydroxy-EB, C-9 epimers of 9-deoxo-9-hydroxyerythronolide B; ethyl-GEB, 3β-[2-(diethylamino)ethoxy]androst-5-en-17-one; $CHCl_3$, chloroform; EtOAc, ethyl acetate; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; G-6-P, glucose 6-phosphate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; FPLC, fast protein liquid chromatography; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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NaB^3H_4, sodium [1-^{14}C]propionate, and Aquasol were from New England Nuclear, Boston, MA; Noble agar was from Difco, Detroit, MI; Bio-Gel P-150 and Bio-Gel HT were from Bio-Rad, Richmond, CA; DEAE-Sepharose CL-6B and the Mono Q and Superose 6 FPLC columns were from Pharmacia, Piscataway, NJ; dialysis membrane was from Union Carbide, Chicago, IL; membrane discs with molecular weight cut-off of 1000 were from Spectrum Medical Ind., Los Angeles, CA; HPLC solvents were from Burdick and Jackson, Muskegon, MI, and J. T. Baker Chemical Co., Phillipsburg, NJ. 6D, EB, and (9*R*)- and (9*S*)-9-deoxo-9-hydroxy-6D and -EB were obtained from Abbott Laboratories, Abbott Park, IL; ethyl-GEB was obtained from the Upjohn Co., Kalamazoo, MI.

Bacterial Growth. Spores of the *S. erythraea* CA 340 or UW22 (same as the NRRL 2338 strain) strain, obtained as described (Weber et al., 1985), were grown on the surface of dialysis membrane overlaying MI-102 medium (Corcoran, 1975) containing 2% agar. Liquid cultures of these strains were grown in the vegetative and fermentation media described by Corcoran (1975). Mycelial cells were harvested by scraping off the dialysis membranes or filtration from the liquid broth and stored at −80 °C until enzyme isolation.

Time Course of EB Production. Liquid or solid cultures were grown, respectively, at 30 °C in a rotary shaker (50 mL of medium/500-mL Erlenmeyer flask fitted with a foam plug) at 200 rpm or in a static incubator. Samples of the growth media were recovered every 24 h during a 6-day period, and EB production was quantitated by TLC and HPLC analysis of EtOAc extracts of the media as described below for liquid cultures and by Weber et al. (1985) for solid cultures.

Preparation of [1,3,5,7,9, or 12-^{14}C]-6D. Following the procedure of Corcoran and Vygantas (1982), the CA 340 strain was grown in the MI-102 medium (50 mL) containing ethyl-GEB (30 mg/L) for 72 h. The mycelia were recovered by centrifugation (2000 rpm, 5 min) and transfected to fresh MI-102 medium (50 mL) containing ethyl-GEB (30 mg/L) and sodium [1-^{14}C]propionate (50 μCi, 56.6 mCi/mmol). After 16 h of growth, the mycelia were removed by centrifugation (5000 rpm, 15 min), and the culture broth was extracted with EtOAc (3 × 50 mL). The combined EtOAc extracts were concentrated under vacuum, and the crude radioactive 6D was isolated by TLC of the resulting residue on thick-layer silica gel plates developed in $CHCl_3$–95% EtOH (10:1). The material was eluted with $CHCl_3$, the $CHCl_3$ extract washed with water and evaporated, and the pure [^{14}C]-6D (6×10^{-3} μCi/mg) isolated from the resulting residue by HPLC according to Tsuji and Goetz (1978) with acetonitrile–methanol–water–0.1 M NH_4OAc (450:50:400:100) as the eluting solvent and detection of the UV absorbance at 214 nm.

Synthesis of (9R)- and (9S)-[9-^{3}H]-9-Deoxo-9-hydroxy-6D. A solution of 6D (4 mg) in methanol (1 mL) was gradually added to a magnetically stirred solution of NaB^3H_4 (340 mCi/mmol) in methanol (1 mL) at −20 °C. After 3 h, the mixture was allowed to warm to 0 °C and left overnight. It was neutralized with cold 10% aqueous NH_4Cl and extracted with EtOAc (3 × 5 mL), and the combined EtOAc extracts were evaporated under vacuum. The two C-9 epimers of the radioactive 9-deoxo-9-hydroxy-6D were isolated from the resulting residue by TLC and HPLC, as described above, and identified by comparison with authentic reference standards.

Isolation of the P-450$_I$ and P-450$_{II}$ 6D Hydroxylases. (All of the following procedures were carried out at 4 °C in a cold room or on ice.) Mycelial cells (100 g, the amount usually recovered from 200 10-cm-diameter Petri dishes) were washed with 200 mL of 0.1 M potassium phosphate buffer (pH 7.3) 3 times and suspended in 200 mL of buffer A [0.1 M potassium phosphate buffer (pH 7.3) containing 1 mM EDTA, 0.2 mM DTT, 0.2 mM PMSF, 1 mM benzamidine, 0.5 mM *p*-aminobenzamidine, and 10% (v/v) glycerol]. The cells were disrupted by homogenization with glass beads in a Bead Beater (Bead Beater, Bartlesville, OK) for 2 min in 30-s intervals with chilling of the homogenizing vessel on ice during the four intervals. The cell homogenate was centrifuged (48000*g*, 30 min) and the supernatant saved. The pellet was suspended in buffer A (200 mL) and sonicated for 2 min at 30-s intervals with intermittant cooling on ice by use of a microtip-equipped Branson Model 185 sonifier. This second cell homogenate was centrifuged as before, and the two supernatants were pooled. The crude cell extract was treated with ammonium sulfate, and the material precipitated at 30–90% saturation was recovered by centrifugation (20000*g*, 30 min). This fraction was chromatographed on DEAE-Sepharose to obtain fractions 1–4 by stepwise elution with 0, 0.1, 0.2, and 0.65 M KCl, respectively, in buffer A. Fraction 3 was concentrated by ultrafiltration and dialyzed against 10 mM buffer A. The concentrated solution was chromatographed on a Bio-Gel HT column (16 × 1.7 cm) previously equilibrated with the same buffer to obtain P-450$_I$ and P-450$_{II}$ by stepwise elution with 10 and 500 mM phosphate, respectively. After concentration by ultrafiltration, each hydroxylapatite fraction (3 mg of protein) was subjected to preparative-scale PAGE (see below) run at 35 mA and 4 °C for 2 h. The area in the gel containing the faintly pink heme band was excised and eluted by overnight dialysis of the gel slice against the buffer used in the next purification step. About 50% of the P-450 enzyme was recovered in this way. The remainder was recovered (with some loss due to denaturation) by electroelution of the gel slice in dialysis tubing in buffer A with a 100-mA current overnight. The recovered material was concentrated and dialyzed against 50 mM Tris-HCl (pH 7.5), 0.5 mM $MgCl_2$, 0.2 mM DTT, 0.2 mM PMSF, 0.2% (w/v) Lubrol Px, 0.05% (w/v) sodium cholate, and 10% (v/v) glycerol (buffer B) and chromatographed on a Mono Q FPLC anion-exchange column (5 × 0.5 cm), previously equilibrated with buffer B, at a 30 mL/h flow rate with collection of 0.5-mL fractions. Following the disappearance of UV absorbances at 280 and 416 nm, the column was eluted with a linear gradient from 30 to 500 mM KCl in buffer B; after the return of base-line UV absorption, it was eluted with 1 M KCl until a total of 150 fractions were collected.

6D Hydroxylase Assay. Stock solutions of substrates were made up in 95% EtOH as follows: [^{14}C]-6D, 0.25 μmol/mL (1.2×10^4 cpm ^{14}C/0.25 μmol); (9*R*)-[9-^{3}H]-9-deoxo-9-hydroxy-6D, 2.57 μmol/mL (1.8×10^6 cpm ^{3}H/2.57 μmol); (9*S*)-[9-^{3}H]-9-deoxo-9-hydroxy-6D, 2.57 μmol/mL (8.0×10^5 cpm ^{3}H/2.57 μmol). The assay mixture consisted of 100 mM phosphate buffer (pH 7.3), 60 nmol of FAD, 1.96 μmol of $NADP^+$, 17.7 nmol of G-6-P, and 3 units of G-6-P dehydrogenase in a total volume of 2 mL. 6D (0.25 μmol) or 0.103 μmol of one of the 9-deoxo-9-hydroxy-6D epimers was used as substrate, and 3 mg of protein from DEAE-Sepharose column fractions 1 and 2 and 7.5 mg from fraction 4 were used as the electron-transport system. Spinach ferredoxin–$NADP^+$ reductase (0.096 unit) and spinach ferredoxin (1 nmol) also were added in the standard assay.

The assay was started by addition of substrate (in buffer from which the EtOH had been removed by a stream of nitrogen) and then preincubation for 1 min at 35 °C before initiation of the reaction by addition of the NADPH-generating system. The mixture was shaken vigorously with EtOAc

to terminate the reaction and then extracted with EtOAc (3 × 3 mL). The combined EtOAc extracts were washed with water and evaporated under vacuum, and the resulting residue was purified by TLC and HPLC as described above to isolate EB or 9-deoxo-9-hydroxy-EB. For analysis of radioactive samples, the plastic TLC sheets were cut into small strips at 1-cm horizontal intervals after being developed twice in $CHCl_3$–EtOH (10:1), and the radioactivity in the strips was measured with a Packard Tri-Carb 460 CD liquid scintillation spectrometer using Aquasol (10 mL/vial). The amount of radioactive product formed was calculated by correcting the cpm recovered for the efficiency of the solvent extraction process (typically 75%), as determined in suitable control experiments.

Molecular Weight Determination. The molecular weight of purified P-450$_I$ and P-450$_{II}$ was estimated by gel filtration on a Bio-Gel P150 column (64 × 2 cm) in buffer A and by SDS–PAGE. Bovine serum albumin (M_r 66000), ovalbumin (M_r 45000), carbonic anhydrase (M_r 29000), and cytochrome *c* (M_r 12400) were used as molecular weight standards.

Preparation of Antisera and Antigenicity Analysis. Four New Zealand white rabbits were injected with DEAE-Sepharose column fraction 3 (each injection contained protein equivalent to 0.32 mg of P-450 dissolved in 1.5 mL of 0.15 M NaCl) emulsified with Freund's adjuvant (0.25 mL of complete in the first injection; incomplete in the second and third injections) every 2 weeks for 6 weeks. The emulsion was administered into footpads and subcutaneously at four different sites on the back of each rabbit, and serum was obtained 2 weeks after the last injection.

For the preparation of specific antibodies against SDS-treated P-450$_I$ and P-450$_{II}$, the Coomassie blue stained band was cut from an SDS–PAGE gel and electroeluted as described above into 100 mM NH_4HCO_3 containing 0.1% SDS. This solution was exhaustively dialyzed against 100 mM NH_4HCO_3 and lyophilized; then, 0.075 mg of lyophilized P-450$_I$ or 0.125 mg of P-450$_{II}$ (per injection) was used to prepare antisera as described above.

For Ouchterlony double-diffusion analysis, 0.01 mL each of antigen and antisera was added to 5-mm wells in Noble agar, and the precipitin bands were observed after 48–72 h.

For Western blots, the protein bands in an SDS–PAGE gel were transfered to nitrocellulose (Schleicher & Schull BA 85) by passive diffusion with the following buffer: 10 mL of 1 M Tris-HCl (pH 7.0), 10 mL of 5 M NaCl, 20 mL of 0.1 M EDTA, and 1 mL of 0.1 M DTT in 1 L of water. The filters were treated with P-450$_I$- or P-450$_{II}$-specific antisera and then anti-rabbit IgG–alkaline phosphatase conjugated antibody in the presence of 5-bromo-4-chloro-3-indolyl phosphate according to literature procedures (Mathew, 1984; *Promega Biotech Notes*, 1986).

Bacterial Alkaline Phosphatase Treatment. Samples of P-450$_I$ and P-450$_{II}$ were exhaustively dialyzed against 10 mM Tris-HCl, pH 8, and their P-450 content was measured. To each sample, 2 μL of bacterial alkaline phosphatase (250 units/μL) was added, and the mixture was incubated at 37 °C for 1 h and then dialyzed overnight against the hydroxylapatite column buffer. The resulting samples were chromatographed on hydroxylapatite, and the dithionite-reduced, CO-complexed different spectra of the P-450-containing fractions were measured. Samples of P-450$_I$ and P-450$_{II}$ were subjected to the same procedures but without addition of the phosphatase for the controls.

General Analytical Methods. PAGE was performed according to Laemmli (1970) with 3% stacking and 10% separating gels. For 0.1% SDS–PAGE, samples and sample buffer were first separately heated in boiling water for 2 min. The sample buffer was made up to 2.8 M 2-mercaptoethanol and mixed with the sample; then, the mixture was heated at 65 °C for 30 min to inhibit protease activity. Cytochrome P-450 in the gels was measured by its peroxidase activity according to Thomas et al. (1976). Isoelectric focusing was carried out in a thin layer of 5% polyacrylamide containing 2.25% ampholyte (pH range 3.5–9.5) according to instructions in the Bio-Rad manual. The p*I* of the samples was determined from a plot of mobility vs. pH with eight different Bio-Rad pH standards. Amino acid analyses were done with a Beckman 6300 amino acid analyzer after sample hydrolysis in 5.7 N HCl containing 3% phenol and 1% mercaptoethanol at 110 °C for 24 h. Protein concentration was determined with the Bio-Rad protein assay kit. Optical and UV absorbance spectra were obtained with Perkin-Elmer 559A or Gilford 250 dual-beam spectrophotometers, and samples were dialyzed against 100 mM phosphate buffer (pH 7.3) containing 10% (v/v) glycerol before analysis. The heme content of samples was determined according to de Duve (1948) as modified by Omura and Sato (1964). A value of 32 cm^{-1} mM^{-1} was used for the difference in molar extinction between 557 and 575 nm in the dithionite reduced minus oxidized difference spectrum of the hemochromogen. The cytochrome P-450 content was determined from the dithionite difference spectrum of CO-complexed samples according to published procedures (Matsubara et al., 1976; Omura & Sato, 1964) with the extinction coefficient determined by the hemochrome assay.

Results

Purification of the Two 6D Hydroxylases. By use of the appearance of EB as the indicator of enzyme titer in vivo and examination of solid and liquid growth of *Saccharopolyspora erythraea* for the production of EB as a function of time, 72-h-old mycelia from cultures grown on solid MI-102 medium were found to have the highest EB and lowest erythromycin A content. These conditions were used for the isolation of the two 6D hydroxylases from the CA 340 strain.

Our enzyme purification scheme followed the procedures described by Corcoran and co-workers (Corcoran et al., 1981, 1982) through the DEAE-Sepharose step with the following two changes. (i) Buffer A, used through the hydroxylapatite chromatography step, contained 1 mM benzamidine, 0.5 mM *p*-aminobenzamidine, 0.2 mM PMSF, and 10% glycerol, in addition to 0.2 mM DTT and 1 mM EDTA, to inhibit proteases (Roberts & Leadlay, 1983; Williams et al., 1984) and further stabilize the enzymes. (ii) Mycelial cells were disrupted by glass bead homogenization and sonication rather than by a French press. The ammonium sulfate precipitate obtained at 30–90% saturation of the cell extract showed distinct absorbances at 428 and 448 nm by dithionite difference spectroscopy of a CO-complexed sample. This material was chromatographed on DEAE-Sepharose by stepwise elution with KCl. Fractions 1 (buffer wash) and 2 (eluted with 0.1 M KCl) showed no absorbances at 420 or 450 nm, but elution with 0.2 M KCl (fraction 3) and 0.65 M KCl (fraction 4) resulted in the characteristic absorbances in the dithionite difference spectra of cytochrome P-450 in fraction 3 and cytochrome P-420 in fraction 4. Fraction 3 was then chromatographed on hydroxylapatite, and two cytochrome P-450 fractions, P-450$_I$ and P-450$_{II}$, were recovered in a ratio of 1:0.8 on the basis of their P-450 content by stepwise elution with 10 and 500 mM phosphate, respectively.

Native PAGE and SDS–PAGE of the two P-450 fractions showed a major band with a molecular weight of about 44000.

Table I: Data for Purification of 6D Hydroxylase from *Saccharopolyspora erythreaea* CA 340

fraction	total protein (mg)	total P-450 (mg)	specific content of P-450 (nmol/mg)	6D hydroxylase activity (pmol h^{-1} mg^{-1})[a]	ratio of 6D hydroxylase activity to P-450 content (pmol $nmol^{-1}$ h^{-1})	yield (%)[b]
crude extract	5500.00	5.00	0.02	0.27	13.50	100
ammonium sulfate	1020.00	3.82	0.08	1.29	16.12	76
DEAE-Sepharose (fraction 3)	220.00	3.34	0.35	79.20	226.28	66
hydroxylapatite						
$P\text{-}450_I$	92.00	1.43	0.33	25.70	77.87	28
$P\text{-}450_{II}$	73.41	1.14	0.33	139.82	423.69	22
preparative PAGE						
$P\text{-}450_I$	3.72	0.71	4.70	431.60	91.82	14
$P\text{-}450_{II}$	2.1	0.44	4.60	468.00	101.73	9
FPLC						
$P\text{-}450_I$	0.12[c]	0.10[c]	17.54	1126.00[d]	64.19	2
$P\text{-}450_{II}$	0.05[c]	0.03[c]	15.27	1386.00[d]	90.76	0.7

[a] Assayed as described under Experimental Procedures. [b] Based on total P-450 content. [c] FPLC fraction 55. [d] Experiment was carried out on FPLC pooled fractions 52–57.

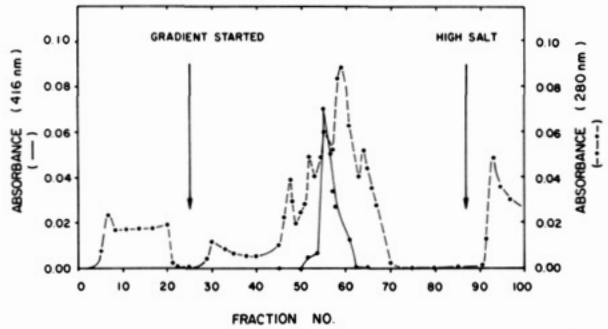

FIGURE 1: Mono Q column elution profile of $P\text{-}450_{II}$ recovered from preparative PAGE. The solid line (A_{416}) shows the absorption of heme-containing fractions, and the dashed line (A_{280}) shows the absorption of protein-containing fractions.

Preparative PAGE was used to further purify the two P-450 hydroxylapatite column fractions by elution of the protein from the gel slice showing positive heme stain and a pinkish color. This procedure resulted in some loss of P-450 material but highly improved the purity of the two enzymes as judged by the lower A_{280} to A_{416} ratios compared with the same values from the previous isolation step.

Complete purification of the $P\text{-}450_I$ and $P\text{-}450_{II}$ 6D hydroxylases was achieved by FPLC of the above material on a Mono Q anion-exchange column in buffer B. The 30–500 mM KCl gradient elution profile (Figure 1) shows that the $P\text{-}450_{II}$ enzyme was eluted in fractions of 52–62. Fraction 55 had a P-450 content of approximately 15.3 nmol/mg of protein as determined by dithionite difference spectroscopy. The $P\text{-}450_I$ enzyme, obtained in the same way, had a P-450 content of 17.5 nmol/mg of protein.

A representative summary of the results of the enzyme isolation scheme is shown in Table I. The final enzyme preparation represents a 2% recovery in $P\text{-}450_I$ and a 0.66% recovery in $P\text{-}450_{II}$ of the P-450 content of the crude cell-free extract.

Purity and Antigenic Relatedness of the Two 6D Hydroxylases. The homogeneity of the final $P\text{-}450_I$ and $P\text{-}450_{II}$ enzyme preparations was verified by three techniques. One major band was observed on SDS–PAGE of both preparations, with $P\text{-}450_{II}$ showing some minor bands in the lower molecular weight range (data not shown). Values of 0.46–0.90 were obtained for the A_{280}/A_{416} ratio, which indicates that the two P-450 proteins were highly purified on the basis of the literature data for other cytochrome P-450 enzymes (Dus et al., 1976). Both preparations formed single precipitin bands when tested against rabbit antisera to DEAE-Sepharose fraction 3 material. (This antisera formed multiple bands when tested against the fraction used for its preparation.) Each preparation also formed a single precipitin band when tested against rabbit antisera raised to either one of the SDS–PAGE-purified P-450 enzymes and showed a complete line of identity in Ouchterlony double-diffusion analysis (data not shown).

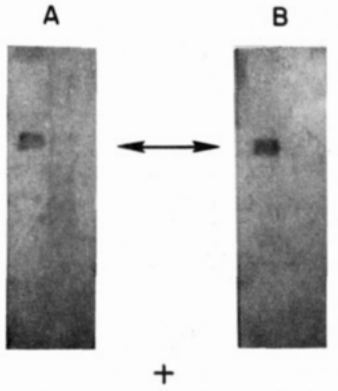

FIGURE 2: Western blot immunodetection analysis of SDS–PAGE-purified $P\text{-}450_I$ with anti-$P450_I$ (A) and anti-$P450_{II}$ (B). SDS–PAGE-purified proteins were used to prepare the antibodies.

The antigenic relatedness of the two 6D hydroxylases was further tested by a Western blot analysis of the $P\text{-}450_I$ enzyme treated with anti-$P\text{-}450_I$ and anti-$P\text{-}450_{II}$ antibodies prepared from SDS–PAGE-purified material. Both antisera reacted similarly with the $P\text{-}450_I$ protein (Figure 2).

Molecular Weight, Isoelectric Point, and Amino Acid Content of the Two 6D Hydroxylases. The mobility of the $P\text{-}450_I$ and $P\text{-}450_{II}$ proteins on SDS–PAGE gels was similar to ovalbumin, indicating a M_r of 44 220 ± 1350 for both enzymes. A similar molecular weight for each enzyme was found by gel filtration with a Bio-Gel P-150 column.

A p*I* of 4.6 was found for these two proteins by isolelectric focusing, and their amino acid analyses, after repurification by SDS–PAGE and isolation from the gels, are shown in Table II. Their amino acid contents are very similar except for small, possibly insignificant, differences in the arginine and proline values.

Enzyme Activity of the Two 6D Hydroxylases. During the enzyme purification, the 6D hydroxylase activity of P-450-containing fractions was determined by a modification of the method of Corcoran and Vygantas (1982) with, as substrates, [^{14}C]-6D prepared biosynthetically, and (9*R*)-[9-^{3}H]-9-deoxo-9-hydroxy-6D prepared by reduction of 6D with Na-B^3H_4. 9-Deoxo-9-hydroxy-6D is an alternate substrate for this

Table II: Amino Acid Compositions of Purified P-450$_I$ and P-450$_{II}$ 6D Hydroxylases and Amino Acid Compositions of Cytochrome P-450 Enzymes from *Pseudomonas putida* (P-450$_{cam}$), *Rhizobium japonicum* (P-450$_{rhi}$), and Rabbit Liver Microsomes (P-450$_{lm}$)

amino acid	P-450$_I$[a]	P-450$_{II}$[a]	P-450$_{cam}$[b]	P-450$_{lm}$[b]	P-450$_{rhi}$[b]
Asx	45	44	36	35	33
Thr	22	19	19	23	14
Ser	17	15	21	26	20
Glx	48	48	55	43	54
Pro	31	26	27	24	25
Gly	36	39	26	30	33
Ala	41	41	34	23	54
Val	30	28	24	27	25
Met	7	4	9	8	8
Ile	18	17	24	19	14
Leu	53	50	40	46	46
Tyr	6	8	9	11	6
Phe	19	19	17	28	17
His	5	6	12	11	12
Lys	9	11	13	19	14
Arg	35	40	24	29	29

[a] To calculate these numbers, the raw data were normalized to the value for Lys, and a M_r of 46 000 was used for the protein. [b] Dus et al., 1976.

enzyme according to Corcoran (1981) and is chemically stabler than 6D.

Reconstitution experiments with DEAE-Sepharose column fractions 1–4 and an NADPH regenerating system established that fraction 3 had a specific activity of 45.6 pmol h^{-1} (mg of protein)$^{-1}$ for the conversion of 6D to EB and 36.3 pmol h^{-1} (mg of protein)$^{-1}$ for the conversion of 9-deoxo-9-hydroxy-6D to 9-deoxo-9-hydroxy-EB. The formation of these compounds was confirmed by large-scale incubations and characterization of the two enzymatic products through comparisons of their mobilities on TLC and HPLC and their low-resolution mass spectral fragmentation patterns with authentic standards.

As reported by Corcoran (1981), spinach cytochrome P-450 reductase and spinach ferredoxin could be substituted for DEAE-Sepharose column fractions 1, 2, and 4 in the enzyme assay. In our hands, this resulted in an approximately 2-fold increase in the hydroxylase specific activity over the original complete assay system, whereas the addition of the same two components to the latter assay system resulted in a 20-fold increase in the specific activity (data not shown). We therefore used (9*R*)-[9-^{3}H]-9-deoxo-9-hydroxy-6D as the substrate along with supplementation of the assay system with spinach cytochrome P-450 reductase and ferredoxin routinely to monitor the 6D hydroxylase activity during enzyme isolation (Table I, column 5). Under these conditions, the rate of hydroxylation was directly proportional to the amount of protein added up to 5.5 mg from DEAE-Sepharose fraction 3; with 2.25 mg of this protein, the rate was first order for nearly 90 min. The K'_m and V'_{max} values for this mixture of 6D hydroxylases were determined from the Lineweaver–Burk reciprocal-plot data and calculated to be 11.5 (±3.2) μM and 121.1 (±16.7) pmol h^{-1} (mg of protein)$^{-1}$, respectively, for the (9*R*)-9-deoxo-9-hydroxy-6D substrate.

The catalytic properties of the two hydroxylapatite column fractions were similarly determined; the K'_m and V'_{max} values of the P-450$_I$ fraction were 12.0 (±2.6) μM and 15.7 (±1.2) pmol h^{-1} (mg of protein)$^{-1}$, respectively, and of the P-450$_{II}$ fraction were 9.0 (±3.3) μM and 193.2 (±24.2) pmol h^{-1} (mg of protein)$^{-1}$, respectively. The difference in the specific activity of these two enzymes with the 9*R* substrate disappeared upon further purification by native PAGE (Table I), but there still was a significant difference with the (9*S*)-[9-^{3}H]-9-deoxo-9-hydroxy-6D substrate: the P-450$_I$ enzyme had a specific activity of 3886.0 pmol h^{-1} (mg of protein)$^{-1}$, nearly 2.5 times greater than that of the P-450$_{II}$ enzyme [1651.0 pmol h^{-1} (mg of protein)$^{-1}$].

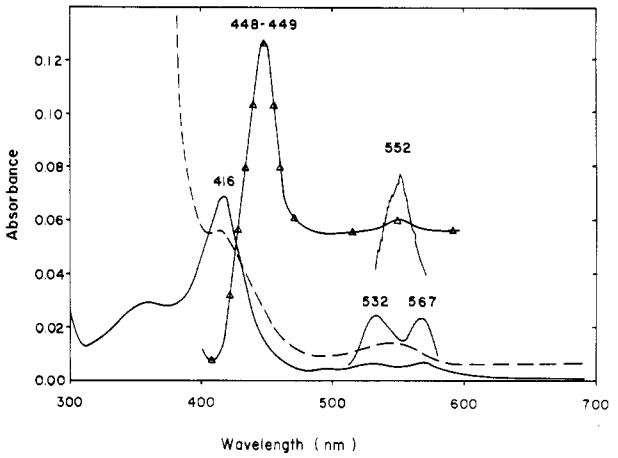

FIGURE 3: Absorption spectra of purified P-450$_I$: (—) oxidized; (--) dithionite reduced; (Δ) dithionite reduced, CO complexed.

The large differences in the V'_{max} between the two 6D hydroxylases recovered from the hydroxylapatite chromatography step indicated that there were factors that affected the reaction velocity differently. Among these, aggregation was discounted because of the identical elution of the two enzymes from a Bio-Gel P-150 column and the lack of any change in their elution behavior by rechromatography of detergent-treated preparations on DEAE-Sepharose, hydroxylapatite, and Mono Q FPLC columns. Their optical absorption spectra (see below) excluded metabolite binding as another possible factor. Loss of heme from the two P-450 proteins could affect their V'_{max} (perhaps differently) due to the presence of enzymatically inactive apoprotein in samples assayed for 6D hydroxylase activity. By expressing the turnover rates of the two enzymes as a ratio of their specific activity to P-450 content (Table I, column 6), it is seen that the highest enzyme activities were after the DEAE-Sepharose and hydroxylapatite steps. This treatment of the data in columns 4 and 5 of Table I, which reinforces the fact that the activities of P-450$_I$ and P-450$_{II}$ became relatively constant upon extensive purification, suggests that concurrent denaturation (leading to loss of activity) and removal of apoprotein (leading to enrichment of heme protein) occurred during enzyme purification.

Optical Properties of the Two 6D Hydroxylases. The absorption spectra of the oxidized, dithionite-reduced and dithionite-reduced CO-complexed P-450$_I$ and P-450$_{II}$ enzymes after their complete purification are shown in Figures 3 and 4. Both of the oxidized preparations showed absorption maxima at 416 (Soret), 532 (β), and 567 nm (α), indicating that the cytochrome P-450 was in the low-spin form in both enzymes. An absorbance peak was also observed at 356 nm in some highly purified preparations, which became more intense on aging of the samples. On reduction, the Soret band was shifted to 409 nm, the 532- and 567-nm absorbances disappeared, and a new band appeared at 543 nm.

Dithionite difference spectroscopy of the CO-complexed cytochrome P-450 produced typical absorption maxima at 448–449 and 552 nm for the P-450$_I$ enzyme and at 449–450 and 552 nm for the P-450$_{II}$ enzyme. A weak absorbance occasionally seen at 422 nm became more intense parallel to a reduction in the intensity of the 448–449- or 449–450-nm absorbances after dialysis against water and lyophilization, which indicates the conversion of P-450 to P-420 by denaturation of the protein. An average extincton coefficient value

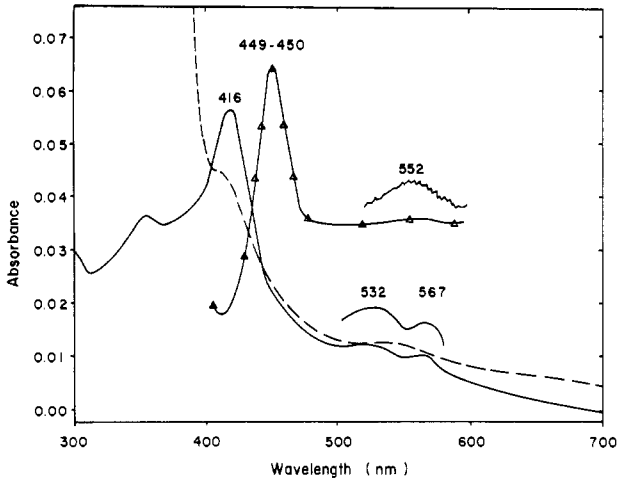

FIGURE 4: Absorption spectra of purified P-450$_{II}$: (—) oxidized; (--) dithionite reduced; (△) dithionite reduced, CO complexed.

of 104.5 ± 4.5 mM^{-1} cm^{-1} was obtained for the P-450$_I$ and P-450$_{II}$ enzymes, on the basis of the differences in the absorbance between 450 and 490 nm and the protoheme content that was calculated by the hemochromogen method (de Duve, 1948; Omura & Sato, 1964).

No evidence favoring the existence of high-spin (Kominami et al., 1980) or metabolite-bound (Kominami et al., 1980; Fisher et al., 1986) cytochrome P-450 was obtained for either enzyme, as indicated by the lack of any significant absorbance at 360–400 and 600–700 nm in the oxidized state. A characteristic absorbance for metabolite-free, dithionite-reduced cytochrome P-450 at 409 and 543 nm was observed in both preparations instead.

Further Tests of the Relationship between the Two 6D Hydroxylases. The possibility of having resolved the two P-450 fractions by hydroxylapatite chromatography on the basis of aggregation or differential sulfhydryl oxidation was eliminated by the following experiments. Samples of the two P-450 fractions were mixed and exhaustively dialyzed against buffer A either containing 0.2% Lubrol Px and 0.05% sodium cholate or containing 10 mM DTT. After centrifugation, the supernatants from each treatment were applied to a hydroxylapatite column that had been equilibrated with the corresponding buffer, and the column was eluted with 10 and 500 mM phosphate. The P-450$_I$ and P-$_{450II}$ fractions were recovered as before with only small losses of material.

DEAE-Sepharose fraction 3 was also chromatographed on a calibrated Bio-Gel P-150 column. The material having an elution volume corresponding to a M_r of 44 000 was collected and rechromatographed on hydroxylapatite in buffer A. The P-450$_I$ and P-450$_{II}$ fractions were again recovered in a ratio similar to that found originally. The same experiment carried out in buffer A lacking glycerol and the protease inhibitors gave similar results, although there was an extensive loss of material. Furthermore, samples of P-450$_I$ and P-450$_{II}$ that had been purified by native PAGE could still be resolved by hydroxylapatite chromatography.

The possibility that resolution of the two 6D hydroxylases was due to differential phosphorylation (Gorbunoff, 1985) was tested by the following experiment. Samples of P-450$_I$ and P-450$_{II}$ that had been purified by FPLC on a Superose 6 column, in addition to the procedures described above, were each treated with bacterial alkaline phosphatase and resubjected to hydroxylapatite chromatography. The elution behavior of the two P-450 enzymes was unchanged, and they were found to have the same dithionite-reduced, CO-complexed difference spectra as before the phosphatase treatment, except for some loss in their P-450 content from denaturation.

Discussion

Both enzymatic activity and cytochrome P-450 content were monitored during purification, and the two forms of 6D hydroxylase, P-450$_I$ and P-450$_{II}$, from *Saccharopolyspora erythraea* CA 340 were resolved on a hydroxylapatite column with consistent reproducibility. At this level of purity, both enzymes showed a typical substrate saturation profile toward (9*R*)-9-deoxo-9-hydroxy-6D with apparent K_m values somewhat higher than the 4-μM value reported for the natural substrate, 6D, with a crude enzyme preparation (Corcoran, 1981). The apparent V_{max} of the form II enzyme is about 12 times higher than that of the form I enzyme. This difference in V_{max} disappeared upon further purification, but the two forms of 6D hydroxylase still exhibited different specific activities toward (9*S*)-9-deoxo-9-hydroxy-6D, and their CO complexes had slightly different dithionite difference spectra. Much faster P-450 denaturation occurred with the form I enzyme than with the form II enzyme when they were incubated at the assay temperature, but after complete purification, the P-450 content of these two enzymes remained constant for at least 2 h under the same conditions.

The completely purified P-450$_I$ and P-450$_{II}$ 6D hydroxylases, on the other hand, have an apparently identical molecular weight, p*I*, amino acid composition, N-terminal sequence for the first five amino acids (data not shown; lack of further sequence data for P-450$_{II}$ prevented a more extensive comparison), and specific activity toward (9*R*)-9-deoxo-9-hydroxy-6D. They also have very similar antigenicity but can still be resolved by hydroxylapatite chromatography, which indicates, consequently, that the reason for this resolvability cannot be due to a major structural difference between the two enzymes.

We speculate that the enzyme in one of the two hydroxylapatite column fractions contains a tightly bound substance, e.g., a phospholipid, which resulted in a greater stability and a higher catalytic activity of the form II enzyme than of the form I enzyme in less purified preparations. This tightly bound material may as well account for the hydroxylapatite chromatography resolution and may not be removed by further purification, but its presence and nature remain to be determined. Alternatively, phosphorylation of the form I enzyme could have caused its lower specific activity and more rapid denaturation to the P-420 form, as precedented for other cytochrome P-450 enzymes (Pyerin et al., 1984, 1987; Taniguchi et al., 1985), in addition to its resolution from the form II enzyme on hydroxylapatite. A preliminary test for protein phosphorylation was negative, but this may have been due to a requirement of a specific phosphorylase as noted in another case (Pyerin et al., 1987).

The two forms of 6D hydroxylase were also isolated from the wild-type UW 22 (=NRRL 2338) strain of *S. erythraea*, which produces much less erythromycin A. The total P-450 content in the crude cell-free extract of this strain was only one-fifth that of CA 340 on the basis of the wet weight of identically aged mycelia.

In the face of scant information about cytochrome P-450 enzymes in the *Streptomyces* (Romesser & O'Keefe, 1986), it is premature to speculate about the physiological significance of the existence of two forms of 6D hydroxylase and their relative concentration in low (UW 22) and high (CA 340) erythromycin-producing strains. Nonetheless, multiple forms of P-450 hydroxylases occur in other microorganisms. In yeast, depending on the growth conditions, a number of different cytochrome P-450 enzymes have been detected, and the sug-

gestion has been made that P-450 synthesis is regulated independently of the other components of the monooxygenase system (Sanglard et al., 1984). For the bacterial enzymes, *Pseudomonos putida* produces only a single P-450 hydroxylase when grown on camphor (Unger et al., 1986) whereas *Rhizobium japonicum* produces at least three forms of P-450 enzyme resolvable by hydroxylapatite chromatography (Appleby, 1978).

It is of possible evolutionary interest to note that the amino acid content of the two 6D hydroxylases from *S. erythraea* is similar to the composition of P-450 enzymes from mammalian liver, *P. putida*, and *R. japonicum* (Table II). Moreover, several of their other characteristics (e.g., optical properties and requirement for a multicomponent electron-transport system) are similar to those for other P-450 enzymes from prokaryotic and eukaryotic organisms.

Further investigation of these two 6D hydroxylase enzymes should establish important information about the regulation of erythromycin biosynthesis. For example, the much smaller apparent K_m values for the P-450$_I$ and P-450$_{II}$ 6D hydroxylases than for the K'_m values reported for propionyl-CoA carboxylase (90 μM; Hunaiti & Kolattukudy, 1982), methylmalonyl-CoA mutase (310 μM; Hunaiti & Kolattukudy, 1984a), and malonyl-CoA decarboxylase (143 μM; Hunaiti & Kolattukudy, 1984b)—three other enzymes that have been suggested to play a role in erythromycin biosynthesis—could have a regulatory significance. The possibility that 6D hydroxylase is inducible by the addition of propanol to the fermentation medium (Petzoldt & Kieslich, 1969) implies that expression of the gene(s) encoding the formation of this enzyme is regulated by the level of the substrates for 6D formation or of 6D itself. Finally, the apparent existence of two forms of 6D hydroxylase in vivo suggests that regulation of 6D hydroxylase activity may involve the posttranslational modification of the P-450 enzyme initially produced. This possibility is being tested in further studies by which we hope to clarify the relationship of these two proteins.

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