Purification and Reconstitution of the Electron Transport Components for 6-Deoxyerythronolide B Hydroxylase, a Cytochrome P-450 Enzyme of Macrolide Antibiotic (Erythromycin) Biosynthesis

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The hydroxylation of 6-deoxyerythronolide B (6D) to erythronolide B, a step in the biosynthesis of the 14-membered macrolide antibiotic erythromycin A by *Saccharopolyspora erythraea*, is catalyzed by a cytochrome P-450 monooxygenase that requires two electron transport proteins for the function of this terminal hydroxylase (A. Shafiee and C. R. Hutchinson, *Biochemistry* 26:6204-6210, 1987). Two flavoproteins and an iron-sulfur protein (erythrodexin) were purified from *S. erythraea* CA340 and shown to act with 6D hydroxylase to catalyze the hydroxylation of (9R)-[9-³H]-9-deoxy-9-hydroxy-6D in vitro in a suitably reconstituted system. The flavoproteins contained flavin adenine dinucleotide and exhibited characteristic absorption maxima at 356 and 456 nm. The one with an M_r of 47,000 showed NADPH-dependent diaphorase and cytochrome *c* reductase activity, and the other, with an M_r of 53,000 showed NADH-dependent activities of the same two types. Erythrodexin contained acid-labile sulfur and iron, had an M_r of 27,500, and showed a broad absorption maximum between 394 and 404 nm. The sequence of its first 15 amino acids, except for position 12, was the same as that of the ferredoxin from *Mycobacterium smegmatis*.

In many living organisms, electron transport-dependent mixed function oxidases catalyze hydroxylation, desaturation, and other oxidative reactions of a large variety of substrates during the metabolism of sterols, fatty acids, xenobiotic compounds, and other small metabolites (6, 22, 23). The cell types involved are known to use two types of systems for the requisite electron transport between the substrate and an NAD(P)H coenzyme, the ultimate electron donor or acceptor. One type consists of an NADPH-dependent reductase alone, or along with a heme protein (cytochrome *b₅*), to supply electrons to the terminal oxidase (10, 23); the other uses an NADPH-dependent reductase plus an iron-sulfur protein (a ferredoxin) for the same process (15). For bacteria, the prototype system is found in *Pseudomonas putida* in which an NADH-cytochrome P-450 reductase and putidaredoxin are required to transfer electrons from NADH to the cytochrome P-450 hydroxylase that acts on camphor (11). The streptomycetes, ubiquitous gram-positive soil bacteria, also contain cytochrome P-450 monooxygenases (19, 21), but definitive information about their associated electron transport components has not been reported.

We recently described (21) the purification and characteristics of a cytochrome P-450 monooxygenase from *Saccharopolyspora erythraea* (formerly *Streptomyces erythraeus*) that catalyzes the hydroxylation of 6-deoxyerythronolide B (6D) to erythronolide B, which is an early step in the biosynthetic pathway to erythromycin A, the clinically important macrolide antibiotic produced by this bacterium. We are studying this hydroxylation as part of our interest in the regulation of erythromycin A biosynthesis at the genetic and biochemical levels. To permit a detailed analysis of the 6D hydroxylation process, we characterized the electron transport components of the 6D hydroxylase system. Their properties and ability to support the hydroxylation of 6D in vitro

in a suitably reconstituted system are reported here, confirming the earlier, preliminary evidence of Corcoran and Vygantas (4, 5) for the role of NADPH-cytochrome P-450 reductase and ferredoxinlike proteins in *S. erythraea*.

MATERIALS AND METHODS

Biochemicals, reagents, and other materials. Phenylmethylsulfonyl fluoride, ammonium sulfate (grade III), benzamidine, *p*-aminobenzamidine, EDTA, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), NADH, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, 2-hydroxybutyrate, 2-hydroxybutyrate dehydrogenase, potassium ferricyanide, cytochrome *c*, bovine serum albumin, carbonic anhydrase, and egg albumin were obtained from Sigma Chemical Co. (St. Louis, Mo.). DEAE-Sepharose CL-6B and Superose 6 fast protein liquid chromatography columns were purchased from Pharmacia, Inc. (Piscataway, N.J.). Dialysis membrane disks with a molecular weight cutoff of 1,000 or 6,000 to 8,000 were purchased from Spectrum Medical Inc. (Los Angeles, Calif.).

Bacterial growth. Spores and mycelial cells of *S. erythraea* CA340 (this strain was obtained from Abbott Laboratories, Abbott Park, Ill.) were prepared as previously described (21).

Purification of NADH and NAD(P)H reductases and an iron-sulfur protein from *S. erythraea*. All the following procedures were done at 4°C in the cold room or on ice. The preparation of cell extracts of mycelial cells, ammonium sulfate precipitation, and DEAE-Sepharose chromatography to obtain the 0.65 M KCl fraction (fraction 4, Fig. 1) by stepwise elution of the column were done as previously described (21).

Fraction 4 was applied to a DEAE-Sepharose column (27 by 2 cm) that had been equilibrated with buffer A (21), and fractions (120 of 6 ml each) were eluted with a linear KCl gradient (0 to 1 M) in the same buffer (1 liter) at a 72-ml/h

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FIG. 1. Flow chart of the steps in the purification of the two NAD(P)H-cytochrome P-450 reductases and an iron-sulfur protein (erythroxin) from *S. erythraea*. K, 10^3 ; ISP, iron-sulfur protein.

flow rate. These fractions were pooled in three groups according to their spectral absorbances at 280 and 455 nm (Fig. 2). Fraction pools B and C were each chromatographed on a molecular weight-calibrated Superose 6 column with a Pharmacia fast protein liquid chromatography unit with buffer B (100 mM Tris hydrochloride [pH 7.5], 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM dithiothreitol, 10% [vol/vol] glycerol) that collected 1-ml fractions at a 15-ml/h flow rate. The fractions eluting between the elution volume of bovine serum albumin and cytochrome *c* were collected; and those with an absorbance of 280 and 455 nm were pooled, concentrated by ultrafiltration through a membrane with a molecular weight cutoff of 1,000, and dialyzed against 50 mM Tris hydrochloride buffer (pH 7) containing 20% glycerol.

Up to 25 mg of total protein (as determined by the Bradford method [2a]) in the dialyzed sample was applied on a 3-mm-thick native polyacrylamide gel (3% stacking, 10% separating) and electrophoresed at 35 mA and 4°C for about 2 h. DEAE-Sephacel pool B gave a band at M_r 47,000 in both native and sodium dodecyl sulfate (SDS)-polyacrylamide gels, and DEAE-Sephacel pool C gave a band at M_r 68,000 in the native polyacrylamide gel but at M_r 53,000 in the SDS-polyacrylamide gel. Both these bands were relatively pure when examined by analytical SDS-polyacrylamide gel electrophoresis (PAGE) (see Fig. 4) and, at high concentrations, exhibited a yellowish color (flavo-

protein). Each flavoprotein band was eluted from native polyacrylamide gels by passive diffusion or by electroelution with a 100-mA current into buffer B overnight at 4°C. The eluted proteins showed absorbances between 300 and 600 nm that are characteristic of flavoproteins.

The iron-sulfur protein (erythroxin) was isolated from DEAE-Sephacel fraction pool C by excising the brown band that had a mobility close to that of cytochrome *c* in a native polyacrylamide gel. The protein eluted from this gel was then run on a 15% polyacrylamide gel, and the purified erythroxin was recovered as described above (see Fig. 4).

Preparation of 6D hydroxylase and (9R)-9-[3 H]-9-dihydro-6D. 6D hydroxylase and (9R)-9-[3 H]-9-dihydro-6D were prepared as previously described (21).

Diaphorase activity. The diaphorase activity of column fractions was determined by using potassium ferricyanide as the electron acceptor and 0.1 ml of sample solution. The assay was done in a 1-ml total volume containing 79 mM potassium phosphate buffer (pH 7.4) and 0.3 mM potassium ferricyanide and started by adding 0.01 ml of 20 mM NADPH or NADH to the reaction mixture. The initial rate of reduction of potassium ferricyanide was measured at 420 nm, and the activity was expressed as micromoles per minute per milligram of protein in the sample solution, using $1.02 \text{ mM}^{-1} \text{ cm}^{-1}$ as the difference extinction coefficient (25). In a separate experiment, the nonspecific reduction of the electron acceptor was determined in the absence of the sample solution, and the value determined was subtracted from the experimental value.

Cytochrome *c* reductase activity. Reduction of cytochrome *c* was measured in a 1-ml total volume containing 200 mM potassium phosphate (pH 7.5), 0.08 mM EDTA, 0.04 mM cytochrome *c*, and 0.1 ml of sample solution. The assay was started by adding 0.01 ml of 20 mM NADPH or NADH to the reaction mixture. The initial rate of reduction was measured at 550 nm, and the activity was expressed as nanomoles per minute per milligram of protein in the sample solution, using $19.6 \text{ mM}^{-1} \text{ cm}^{-1}$ as the difference extinction coefficient for cytochrome *c* (20). The experimental data were corrected as described above for the diaphorase activity.

Analysis of acid-labile iron-sulfur protein. Acid-labile sulfur was determined in crude and purified protein samples by

FIG. 2. Elution profile of fractions with 280- and 455-nm absorbances from the DEAE-Sephacel column eluted with a 0 to 1 M KCl gradient. B and C designate the fraction pools from which the NAD(P)H-cytochrome P-450 reductases and erythroxin of *S. erythraea* were isolated.

methylene blue formation by the method of Beinert (1). Sodium sulfide was used to establish a standard curve by which the acid-labile sulfur content of samples was quantitated. Acid-labile iron was determined by the method of Massey (13).

Flavin analysis. Purified protein samples were assayed for the presence of FAD and FMN by thin-layer chromatography and high-performance liquid chromatography (18).

Cytochrome *b₅* analysis. The method of Omura and Sato (16) was used to assay purified protein samples for the presence of cytochrome *b₅*. Briefly, sodium dithionite or NADH was added to a sample containing an NADH-dependent reductase at 25°C, and then the mixture was scanned between 400 and 500 nm against a reference sample for any change in the difference absorption spectrum.

Reconstitution of 6D hydroxylase activity. Enzymatically active preparations were reconstituted in 2.5 ml of 100 mM phosphate buffer (pH 7.3) which contained 25 µg of FAD, 25 µg of FMN, 10.6 mg of cytochrome P-450 protein from fraction 3 of the DEAE-Sepharose column (21), 200 µg of iron-sulfur protein, and 175 µg of the *M_r*-47,000 NAD(P)H-dependent reductase or 500 µg of the *M_r*-53,000 NADH-dependent reductase, plus 10 µg of substrate (from a stock solution of radioactive (9*R*)-[9-³H]-9-dihydro-6D having a specific radioactivity of 1.46×10^7 cpm/mg per ml [5.89×10^6 cpm/µmol]). The reaction was started by adding an NADPH-generating system consisting of 1.5 mg of NADP, 5 mg of glucose-6-phosphate, and 3 U of glucose-6-phosphate dehydrogenase or an NADH-generating system consisting of 1.4 mg of NADH, 2.52 mg of 2-hydroxybutyrate, and 0.5 U of 2-hydroxybutyrate dehydrogenase. The mixture was incubated at 33°C for 1 h and worked up as previously described (21).

General assay procedures. Spectroscopic determinations were performed at 25°C with a Perkin-Elmer 559A double-beam UV/VIS spectrophotometer. The determination of molecular weight and protein concentration and analytical SDS-PAGE were done as previously described (21). SDS-polyacrylamide gels were silver stained by the procedure provided by Bio-Rad Laboratory (Richmond, Calif.). Protein N-terminal amino acid sequencing and amino acid analyses were done at Harvard University in the Department of Cellular and Developmental Biology.

RESULTS

Purification of NADH- and NADPH-cytochrome *c* reductases from *S. erythraea*. Since *S. erythraea* CA340 has a low titer of 6D hydroxylase, which has low specific activity (21), it was not feasible to isolate the requisite electron transport proteins, NAD(P)H-cytochrome P-450 reductase(s), and the iron-sulfur protein, erythrodexin, by assaying the hydroxylation of radioactive 6D in a suitable reconstitution system. We therefore decided to follow the purification of the desired NAD(P)H reductases using two different artificial electron acceptors, potassium ferricyanide and cytochrome *c*, which have been routinely used to assay such enzymes in mammalian liver cells (15). The enzymes isolated by these assays usually have proved to be the physiological catalysts for transferring reducing equivalents to terminal monooxygenases (15).

Following the method for our purification of 6D hydroxylase (21), which is summarized in Fig. 1, we found diaphorase and cytochrome *c* reductase activities in DEAE-Sepharose column fractions 2, 3, and 4, with the major portion being detected in fraction 4. The proteins in this fraction

therefore were subjected to further purification by DEAE-Sepharose chromatography with a linear KCl gradient to elute the column. Its elution profile (Fig. 1) shows three pools of fractions. No diaphorase or cytochrome *c* reductase activity was detected in pool A, but it contained all the cytochrome P-420 produced from denaturation of the cytochrome P-450 protein (the 6D hydroxylases [21]); both NADH- and NADPH-dependent diaphorase activity was detected in pool B; and only NADH-dependent diaphorase activity was seen in pool C. Pools B and C contained cytochrome *c* reductase activity as well as iron-sulfur protein. As found for the diaphorase activity, pool B contained NADH- and NADPH-dependent cytochrome *c* reductases with specific activities of 7.9 and 5.4 nmol/min per mg of protein, respectively, whereas pool C had only an NADH-dependent reductase with a specific activity of 25 nmol/min per mg of protein.

Fraction pools B and C were separately chromatographed through a gel filtration column, and the fractions from each that had absorption maxima at 356 and 455 nm were pooled and purified further by preparative PAGE. After electrophoresis on a native protein gel, cuts were made at 5-mm intervals along the length of the gel, corresponding to a range of molecular weight standards from 12,000 to 70,000, and the protein in each gel slice was eluted. Each eluant was assayed for diaphorase and cytochrome *c* reductase activities and scanned between 250 and 600 nm. A protein with an *M_r* of 47,000 and typical flavoprotein absorbance maxima at 356 and 456 nm (Fig. 3, peak A) was thus isolated from the DEAE-Sepharose fraction pool B. This preparation showed one major band of *M_r* 47,000 in Coomassie blue- or silver-stained SDS-polyacrylamide gels (Fig. 4). Fraction pool C similarly yielded a highly purified protein with an *M_r* of 68,000 on native polyacrylamide gel, but with an *M_r* of

FIG. 3. (A) Visible light absorption spectrum of the PAGE-purified *M_r*-47,000 flavoprotein from *S. erythraea* with NAD(P)H-cytochrome P-450 reductase activity. (B) Visible light absorption spectrum of the PAGE-purified *M_r*-53,000 flavoprotein from *S. erythraea* with NADH-cytochrome P-450 reductase activity.

FIG. 4. SDS-polyacrylamide gel of the purified electron transport proteins from *S. erythraea* after Coomassie blue staining. Lanes: A, molecular weight standards (bovine serum albumin, 66,000; egg albumin, 45,000; carbonic anhydrase, 29,000; cytochrome *c*, 12,400); B, NADH-cytochrome P-450 reductase, M_r 53,000; C, NAD(P)H-cytochrome P-450 reductase, M_r 47,000; D, erythrodexin, M_r 27,500.

53,000 on SDS-polyacrylamide gel (Fig. 4), that showed typical flavoprotein absorption maxima at 356 and 456 nm (Fig. 3, peak B). Both these proteins contained FAD but no FMN when assayed as described in Materials and Methods.

A summary of the diaphorase activity observed during purification of these two proteins is shown in Table 1. The cytochrome *c* reductase activity was measured with enzymes purified by preparative PAGE, which showed that the M_r -47,000 protein had both NADH-dependent reductase activity (5.00 nmol/min per mg of protein) (which could be due to contamination by the M_r -53,000 enzyme [Fig. 4]) and NADPH-dependent reductase activity (9.37 nmol/min per mg of protein) and that the M_r -53,000 protein had only NADH-dependent reductase activity (32.00 nmol/min per mg of protein) in the presence of the purified erythrodexin (see below) that was added to the assay mixture. Addition of erythrodexin stimulated the rate of cytochrome *c* reduction by the completely purified M_r -47,000 protein, but not by the M_r -53,000 protein, yet both purified enzymes exhibited a significant amount of erythrodexin-independent reduction of cytochrome *c* (data not shown).

TABLE 1. Purification of NAD(P)H-dependent reductases from 80 g of *S. erythraea* CA340 mycelia by diaphorase activity

Fraction	Total protein ^a (mg)	Sp act ^b	
		NADH	NADPH
Crude cell extract	1,275.00	0.160	ND ^c
Ammonium sulfate precipitate	770.00	0.350	0.042
DEAE-Sepharose			
Fraction 1 (0.0 M KCl)	60.00	0.000	0.000
Fraction 2 (0.10 M KCl)	63.25	0.022	0.017
Fraction 3 (0.20 M KCl)	143.00	0.080	0.013
Fraction 4 (0.65 M KCl)	187.50	0.084	0.024
DEAE-Sepharose (gradient)			
Fraction pool A	35.00	0.000	0.000
Fraction pool B	7.79	0.100	0.200
Fraction pool C	6.23	2.070	0.000
Preparative PAGE			
Fraction pool B ^d	2.20	1.470	3.900
Fraction pool C ^d	3.25	13.30	0.000

^a Determined by the method of Bradford (2a).

^b Micromoles per minute per milligram of protein.

^c ND, Not determined.

^d From the DEAE-Sepharose gradient column.

TABLE 2. Purification of the iron-sulfur protein (erythrodexin) from 200 g of *S. erythraea* mycelia

Fraction	Total protein (mg) ^a	Total acid-labile S (mg) ^b
Crude cell extract	5,200.00	ND ^c
Ammonium sulfate precipitate	2,125.00	0.302
DEAE-Sepharose		
Fraction 1 (0.00 M KCl)	200.00	0.000
Fraction 2 (0.10 M KCl)	240.00	0.015
Fraction 3 (0.20 M KCl)	368.00	0.019
Fraction 4 (0.65 M KCl)	360.00	0.250
DEAE-Sepharose (gradient)		
Fraction pool A	117.50	0.000
Fraction pool B	15.00	0.002
Fraction pool C	12.00	0.058
Preparative PAGE (fraction pool C ^d)	1.20	0.018

^a Determined by the method of Bradford (2a).

^b Determined by the method of Beinert (1).

^c ND, Not determined.

^d From the DEAE-Sepharose gradient column.

Purification of an acid-labile iron-sulfur protein (erythrodexin) from *S. erythraea*. As summarized in Table 2, erythrodexin was purified from DEAE-Sepharose fraction pool C by assaying for acid-labile iron-sulfur protein by the method of Beinert (1). Successive purification by native PAGE with 10% and then 15% running gels gave an intensely brown band near the solvent front that on elution resulted in a highly purified protein of M_r 27,500 on SDS-PAGE (Fig. 4) with a broad absorption maximum between 394 and 404 nm (Fig. 5). This protein gave a positive result when assayed for the presence of acid-labile iron and the following N-terminal amino acid sequence: Thr-Tyr-Val-Ile-Ala-Glu-Pro-Cys-Val-Asp-Val-Leu-Asp-Lys-Ala. Analysis of its amino acid composition revealed 15 aspartic acid and 21 glutamic acid residues per mole.

Reconstitution of the 6D hydroxylase system in vitro. As previously reported (21), (9*R*)-[9-³H]-9-dihydro-6D was used

FIG. 5. Visible light absorption spectrum of the PAGE-purified M_r -27,500 erythrodexin from *S. erythraea*.

as an alternate substrate to improve the sensitivity of the 6D hydroxylase assay in vitro. When assayed as described in Materials and Methods, the NADPH-dependent reductase (M_r 47,000) catalyzed hydroxylation of this substrate in the presence of erythroxin and partially purified 6D hydroxylase and exhibited a specific activity of 4.80 pmol/h per mg of protein (Table 3). Supplementation of the assay reaction with an NADH-generating system resulted in an increase in this specific activity (6.96 pmol/h per mg of protein), possibly due in part to the contaminating M_r -53,000 enzyme. Using the same assay procedure but with the M_r -53,000 NADH-dependent reductase resulted in substrate hydroxylation with a specific activity of 5.80 pmol/h per mg of protein (Table 3). As before, when this assay reaction was supplemented with an NADH-generating system, the specific activity increased (12.96 pmol/h per mg of protein). Elimination of any component of the monooxygenase system from either assay reaction abolished all the enzymatic activity.

DISCUSSION

In our purification of two forms of a cytochrome P-450 protein from *S. erythraea* that catalyzes the hydroxylation of 6D or its alternative substrate, 9-dihydro-6D, we used other fractions from the purification process (Fig. 1) as the source of the electron transport components of this monooxygenase system to reconstitute 6D hydroxylase activity in vitro. Since this activity could be stimulated by the addition of NADPH-cytochrome *c* reductase and ferredoxin from spinach to the assay reaction, we assumed that equivalent proteins were present in *S. erythraea*. Thus, in the present work, we used well-established biochemical assays for NAD(P)H-dependent reductases and iron-sulfur proteins to isolate highly purified proteins that can transport electrons from NADH or NADPH to the terminal 6D hydroxylase in a suitably reconstituted in vitro system.

The two nucleotide-dependent reductases are flavoproteins like many other enzymes that can reduce cytochrome P-450-based monooxygenases (17). But they differ in which nucleotide coenzymes provide the reducing equivalents to the terminal oxidase. The M_r -47,000 cytochrome P-450 reductase principally depends on NADPH as well as erythroxin. It therefore is identical to the cytochrome P-450 monooxygenase systems of the mitochondria of adrenal cortex cells (3) and *Bacillus megaterium* (2) (the NADPH-dependent reductase of the latter contains FMN, however) which use NADPH and an iron-sulfur protein. The M_r -53,000 reductase mainly depends on NADH (for the reduction of cytochrome *c* more than for the reduction of the cytochrome P-450 6D hydroxylase), plus erythroxin, and therefore is identical to the cytochrome P-450 (camphor)

hydroxylase system of *P. putida* (11). It is plausible that both the electron transport-dependent reductases function in *S. erythraea*, at the same time or at different stages of bacterial development, depending perhaps on the time of erythromycin A production.

We speculate that the enhancement of 6D hydroxylase activity upon the addition of the second nucleotide (NADH) to assay reactions containing one or the other of these reductases is due to facilitation of the second electron transfer to the cytochrome P-450 as suggested by Hildebrandt and Estabrook (9) (reviewed by Peterson and Prough [17]). They suggested that the second electron transfer is carried out through cytochrome *b*₅. Since we could not detect cytochrome *b*₅ in the preparations used for the reconstitution of 6D hydroxylase activity, it is possible that in *S. erythraea* erythroxin substitutes for the function of cytochrome *b*₅. The fact that cytochrome *b*₅ can functionally replace putidaredoxin in the camphor hydroxylase system in vitro (7) justifies the latter suggestion.

Erythroxin, whose role as an electron transport protein in *S. erythraea* was proven by the results of the cytochrome *c* reduction and 6D hydroxylase experiments, has a pI of <4 and was eluted from the DEAE-Sepharose column at high salt concentration at pH 7.3. These data show that it is an acidic protein, consistent with its high content of acidic amino acids, and similar to other iron-sulfur proteins that function in electron transport such as adrenodoxin (14) and putidaredoxin (24). Its N-terminal amino acid sequence was identical to that of the ferredoxin from *Mycobacterium smegmatis* (8) for the 15 residues determined except for the substitution of lysine for leucine at position 12. The M_r of erythroxin is 27,500, while adrenodoxin and putidaredoxin have relative molecular weights of 12,500 and 13,100, respectively (7, 14). We cannot at this time offer a reason for the much larger apparent molecular weight of erythroxin, but note that a cytosolic adrenodoxin preprotein of M_r 19,500 has been reported recently by Okamura et al. (14). Furthermore, we cannot rule out the presence of an acid-stable iron-sulfur protein in *S. erythraea* similar to what has been reported for *Pseudomonas oleovorans* (12), which functions as an electron transport component in the ω -hydroxylation of fatty acids and has a molecular weight of 19,500.

The results presented here all indicate that we identified and purified the electron transport components involved in 6D hydroxylation in vivo, but we cannot rule out the participation of other proteins in the actual biosynthetic process by which 6D is converted to erythronolide B in *S. erythraea*. Further insight into this matter must await the outcome of genetic and biochemical studies of erythromycin biosynthesis currently under way in our laboratory.

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TABLE 3. Hydroxylase activity determined with the purified M_r -47,000 and M_r -53,000 NAD(P)H-cytochrome P-450 reductases

Expt	Assay component	Sp act ^a	
		M_r 47,000	M_r 53,000
1	Complete ^b	4.80	5.80
2	+ NADH	6.96	12.96
3	- Cytochrome P-450 protein ^c	0.00	0.00
4	- NAD(P)H-cytochrome <i>c</i> reductase	0.00	0.00
5	- Erythroxin	0.00	0.00

^a Picomoles per hour per milligram of protein.

^b Defined in Materials and Methods.

^c The 9-dihydro-6D hydroxylase (21).

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