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Purification and Characterization of a Soybean Flour-Induced Cytochrome P-450 from *Streptomyces griseus*

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A soybean flour-induced, soluble cytochrome P-450 ($P\text{-}450_{soy}$) was purified 130-fold to homogeneity from *Streptomyces griseus*. Native cytochrome $P\text{-}450_{soy}$ is a single polypeptide, with a molecular weight of 47,500, in association with one ferriprotoporphyrin IX prosthetic group. Oxidized $P\text{-}450_{soy}$ exhibited visible absorption maxima at 394, 514, and 646 nm, characteristic of a high-spin cytochrome P-450. The CO-reduced difference spectrum of $P\text{-}450_{soy}$ had a Soret maximum at 448 nm. When reconstituted with spinach ferredoxin and spinach ferredoxin:$NADP^+$ oxidoreductase, purified cytochrome $P\text{-}450_{soy}$ catalyzed the NADPH-dependent oxidation of the xenobiotic substrates precocene II and 7-ethoxycoumarin. In vitro proteolysis of cytochrome $P\text{-}450_{soy}$ generated a stable and catalytically active cytochrome P-450, designated $P\text{-}450_{soy\Delta}$.

Streptomyces griseus (ATCC 13273) has the remarkable ability to catalyze the stereo- and regiospecific oxidation of a diverse array of xenobiotics. The types of reactions performed by *S. griseus* include aromatic, cyclic, and aliphatic hydroxylations, O-, S-, and N-oxidations, C-C fission, epoxidation, and O- and N-dealkylations. These transformations, which occur with compounds such as alkaloids (17, 27), coumarins (25), rotenoids (28), chromenes (30), and other complex xenobiotics (7, 26), parallel those performed by mammalian cytochromes P-450 (34, 36). However, the nature of the enzymatic system(s) employed by *S. griseus* for such diverse reactions has remained unresolved (26). The biotransformations catalyzed by *S. griseus* occur primarily following growth of the organism on a complex medium enriched with soybean flour (26). Recently, Sariaslani and Kunz (29) demonstrated that soybean flour and one of its isoflavonoid constituents, genistein, induce the synthesis of cytochrome P-450 in *S. griseus* and inferred a role for this enzyme in the biotransformation reactions performed by this organism. Here we report the purification and characterization of a soybean flour-induced cytochrome P-450 and demonstrate the transformation of two xenobiotic substrates, precocene II and 7-ethoxycoumarin, by purified preparations of this enzyme.

(A preliminary description of these results was presented at the 87th Annual Meeting of the American Society for Microbiology [M. K. Trower, D. P. O'Keefe, and F. S. Sariaslani, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, abstr. O6, p. 261]. It should be noted that the enzymes designated cytochromes $P\text{-}450_{SG1}$ and $P\text{-}450_{SG2}$ in the previous account represent cytochromes $P\text{-}450_{soy\Delta}$ and $P\text{-}450_{soy}$, respectively, in the present communication.)

MATERIALS AND METHODS

Microorganism, growth, harvest, and general protein procedures. *S. griseus* ATCC 13273 was grown and harvested as described previously (29) except that the concentration of the soybean flour in the medium was increased to 25 g/liter. Pelleted cells were stored at −80°C until required. Protein contents were estimated by the procedures of Gornall et al. (11) and Bradford (6). The cytochrome P-450 concentration was determined by the method of Omura and Sato (23). Heme was analyzed as its reduced pyridine hemochrome derivative (10). High-pressure liquid chromatography (HPLC) was performed on a system consisting of an LKB 2152 controller, LKB 2150 pump, and a Hewlett-Packard 1040A diode array detector.

Cytochrome $P\text{-}450_{soy}$ purification protocol. All procedures except steps 4 and 5 were carried out at 4°C. Glycerol (20% [vol/vol]) was included in all buffers following the salt precipitation step to prevent degradation of the P-450 to its inactive P-420 species. Protease inhibitors used in steps 1 to 3 to inhibit a wide range of protease activities were pepstatin (an acid protease inhibitor), leupeptin (a broad-range protease inhibitor), and phenylmethylsulfonyl fluoride (PMSF; a serine protease inhibitor). Fractions were stored between purification steps at −80°C with no discernable loss of P-450 specific content.

Step 1: preparation of crude cell extract. Frozen cells of *S. griseus* (approx. 250 to 350 g, wet weight) were thawed in 100 mM sodium phosphate buffer, pH 7.4, containing 1.0 mM dithiothreitol and 0.1 mM EDTA containing the protease inhibitors pepstatin (0.7 mg/liter) and leupeptin (0.5 mg/liter) at 1.5 ml of buffer per g of cells. Cell extracts (105,000 × *g*) were prepared as described before (29) except that cells were disrupted by a single passage through a French pressure cell. PMSF (0.5 mM) was added to the slurry following cell disintegration.

Step 2: ammonium sulfate fractionation. The 105,000 × *g* supernatant was fractionated by collecting the ammonium sulfate precipitate between 30 and 50% saturation by centrifugation (18,000 × *g*, 20 min) and then redissolving in a small volume of PEGP buffer (100 mM sodium phosphate [pH 7.2], containing 1.0 mM EDTA, 20% [vol/vol] glycerol, 0.2 mM PMSF, 0.7 mg of pepstatin per liter, and 0.5 mg of leupeptin per liter).

Step 3: gel filtration chromatography. The resuspended protein (approx. 1.0 g) was applied to a Sephacryl S-200 HR column (5 by 70 cm) and eluted (70 ml/h) with PEGP buffer. Collected fractions (15 ml) containing P-450 were pooled (designated S1) and concentrated to a final volume of 5 to 10 ml by ultrafiltration (Amicon PM-30).

Step 4: anion-exchange HPLC. The S1 fraction was chromatographed on a DEAE-3SW column (7.5 by 150 mm) with

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TABLE 1. Purification protocol for P-450$_{soy}$ and P450$_{soy\Delta}$

Step	Total protein (mg)	Total activity (nmol of P-450)	Specific content (nmol of P-450/mg of protein)	Recovery (%)	Purification (fold)
105,000 × *g* supernatant (309 g, wet weight)	3,170	381	0.12	100	1.0
Ammonium sulfate (30–50%)	910	325	0.36	85	3.0
Sephacryl S-200 HR	153	200	1.3	53	10.8
DEAE-3SW	6.6	83	12.6	21.8	105
S-type hydroxylapatite					
P-450$_{soy}$	1.3	20.4	15.7	5.4	131
P-450$_{soy\Delta}$	1.5	23.5	15.7	6.2	131

buffer A (20 mM Tris-acetate [pH 7.4] containing 20% [vol/vol] glycerol) and buffer B (0.8 M sodium acetate in buffer A) (16). Proteins were eluted (0.75 ml/min) with the gradient 15% B (0 to 5 min), 15 to 55% B (5 to 30 min), 55 to 90% B (30 to 40 min), 90% B (40 to 50 min), and 90 to 15% B (50 to 65 min). The resolved P-450 fraction (designated F1), which eluted at 15 to 16 ml, was collected when the 390/280 nm ratio was greater than or equal to 1:1, concentrated, and equilibrated with 10 mM PCG buffer (sodium phosphate buffer [pH 6.8] containing 0.01 mM $CaCl_2$ and 20% [vol/vol] glycerol) with an Amicon Centricon-30 concentrator.

Step 5: hydroxylapatite HPLC. The F1 fraction was chromatographed on a Regis KB S-type hydroxylapatite HPLC column. Proteins were eluted (0.5 ml/min) with a 30-min linear gradient of PCG buffer from 10 to 100 mM. Two cytochrome P-450 fractions, designated P-450$_{soy}$ and P-450$_{soy\Delta}$, were resolved at 4.5 to 6.0 and 10.5 to 11.0 ml, respectively; these were collected when the 390/280 nm ratio was greater than or equal to 1.0:1.5 and then concentrated as described above.

LDS-PAGE. Lauryl dodecyl sulfate-polyacrylamide gel electrophoresis (LDS-PAGE) was carried out on an 8% resolving gel with the discontinuous system described by Laemmli (18). Molecular weight markers used were phosphorylase *b* (97,400), bovine serum albumin (68,000), egg albumin (43,000), carbonic anhydrase (29,000), chymotrypsinogen (25,700), β-lactoglobulin (18,400), and lysozyme (14,400).

Preparation of apoproteins, amino acid analysis, and tryptic mapping. P-450 apoproteins were prepared by reverse-phase HPLC (RPHPLC) on a Vydac C_4 column (4.6 by 250 mm). The solvent systems used were solvent A (distilled water containing 0.1% [vol/vol] trifluoracetic acid [TFA]) and solvent B (acetonitrile containing 0.1% [vol/vol] TFA) at a flow rate of 0.75 ml/min with the following gradient: 30% B (0 to 5 min), 30 to 70% B (5 to 35 min), 70 to 90% B (35 to 40 min), 90% B (40 to 50 min), and 90 to 30% B (50 to 55 min). Eluates were monitored for heme at 400 nm and for protein at 220 and 280 nm. The P-450$_{soy}$ and P-450$_{soy\Delta}$ apoproteins were collected and then concentrated under a stream of nitrogen, followed by lyophilization.

P-450 apoproteins were alkylated with iodoacetic acid to generate their carboxymethyl adducts (33) and purified by RPHPLC as described above. The amino acid compositions of these proteins were obtained after 20 h of hydrolysis in 6 N HCl at 110°C, followed by analysis on a Beckman 6300 amino acid analyzer.

For tryptic mapping, 1 nmol of apoproteins evaporated to dryness was suspended in 0.1 mM ammonium bicarbonate buffer, pH 8.0 (300 μl). TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone)-treated trypsin was added (enzyme/P-450 ratio of 1:50) and incubated overnight at room temperature. Peptide maps were obtained by RPHPLC as described above, except that a gradient (1.0 ml/min) of 0 to 70% B (0 to 60 min), 70 to 90% B (60 to 70 min), and 90% B (70 to 80 min) was employed.

Preparation of polyclonal antisera and Western blot analysis. Stained bands corresponding to P-450$_{soy}$ and P-450$_{soy\Delta}$ were excised from a preparative LDS-PAGE gel and homogenized by grinding in liquid nitrogen. Polyclonal antibodies were produced at Hazelton Research Products Inc., Denver, Pa., by emulsifying the homogenized gel bands (containing 200 μg of protein) with Freund complete adjuvant and intradermally injecting the preparations into New Zealand White rabbits. Similarly prepared boosters (100 μg) emulsified with Freund incomplete adjuvant were injected subcutaneously every 2 weeks for a total of 6 weeks and 10 weeks for P-450$_{soy}$ and P-450$_{soy\Delta}$, respectively. Rabbits were bled at two weekly intervals after the third week, and the resulting sera were assayed for specific immunologic activity by Western blot analysis.

Western blot analyses were carried out as described by Towbin et al. (35). Following LDS-PAGE separation, protein bands were electrophoretically transferred to Immobilon membrane filters (Millipore Corp., Bedford, Mass.). The filters were then treated with either anti-P-450$_{soy}$- or anti-P-450$_{soy\Delta}$-specific antisera. Antibody-binding protein bands were visualized by using anti-rabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase as a probe and developed with 4-chloro-1-naphthol (14).

Xenobiotic transformations. The reconstituted reaction mixture (5 ml) contained TES (*N*-tris[hydroxymethyl] methyl-2-aminoethanesulfonic acid) buffer, pH 6.8 (20 mM), P-450 (0.1 μM), either 5 mM precocene II (in ethanol) or 1 mM 7-ethoxycoumarin (in dimethyl sulfoxide), and saturating levels of NADPH (0.72 mM), spinach ferredoxin (7 μM), and ferredoxin:NADPH oxidoreductase (0.75 U). Reaction mixtures were incubated with shaking for 1 h at 30°C, extracted into ethylacetate, and analyzed by both thin-layer chromatography (TLC) (25, 30) and by gas-liquid chromatography.

Gas-liquid chromatography was performed on a Hewlett-Packard model 5890 gas chromatograph equipped with a DB-17 bonded-phase fused silica column (30 m; 0.25-mm internal diameter) with helium (37 lb/in^2) as the carrier gas and injector and flame ionization detector temperatures of 350°C. The oven temperatures for analysis of extracts of precocene II and 7-ethoxycoumarin incubations were 200 and 180°C, respectively. Under these conditions, precocene II and its *cis*- and *trans*-dihydrodiols eluted after 1.9, 7.0, and 7.5 min, respectively, while the elution times for 7-ethoxycoumarin and 7-hydroxycoumarin were 7.6 and 10.0 min, respectively.

Effect of protease inhibitors on P-450 content. Harvested *S. griseus* cells were divided into two batches, and extracts

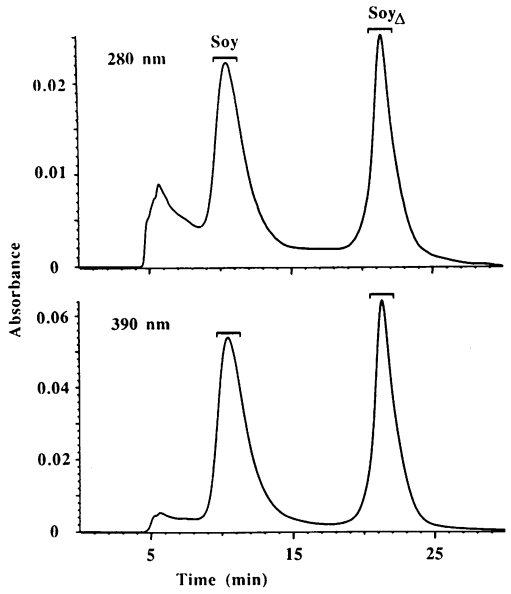

FIG. 1. S-type hydroxylapatite HPLC profiles (280 and 390 nm) of the anion-exhange-purified P-450 fraction (F1) (0.4 mg of protein and 5 nmol of P-450 loaded).

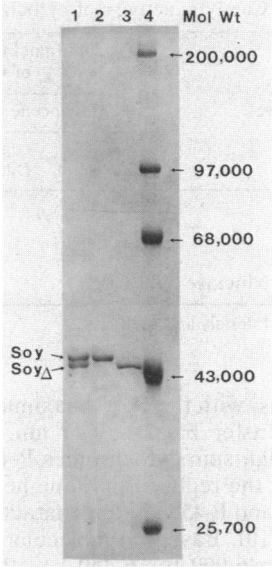

FIG. 2. LDS-PAGE analysis of the purified cytochromes P-450$_{soy}$ and P-450$_{soy\Delta}$ under reduced and denaturing conditions. Lane 1, Mixture of P-450$_{soy}$ (2.0 μg of protein) and P-450$_{soy\Delta}$ (2.0 μg of protein). Lane 2, P-450$_{soy}$ (2.0 μg of protein). Lane 3, P-450$_{soy\Delta}$ (2.0 μg of protein). Lane 4, Molecular weight standards.

were prepared as described above except that only one batch was treated with protease inhibitors. The two extracts were then fractionated with ammonium sulfate and chromatographed on an analytical anion-exchange HPLC column under the conditions described for step 4 of the purification process, except that the following gradient was employed: 15% B (0 to 5 min), 15 to 25% B (5 to 35 min), 25 to 90% B (35 to 45 min), 90% B (45 to 55 min), and 90 to 15% B (55 to 70 min).

Materials. Soybean flour was purchased from Natural Sales Co., Pittsburgh, Pa.; TPCK-trypsin, spinach ferredoxin, spinach ferredoxin:NADP$^+$ oxidoreductase, 7-hydroxycoumarin, PMSF, and TES were bought from Sigma Chemical Co., St. Louis, Mo.; leupeptin and pepstatin were obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind.; anti-rabbit IgG conjugated with horseradish peroxidase and 4-chloro-1-naphthol were purchased from BioRad Laboratories, Richmond, Calif.; *cis*- and *trans*-precocene II dihydrodiols were available from previous studies in this laboratory (30). Other materials were of the finest commercial grade available.

RESULTS

Purification of cytochrome P-450$_{soy}$. Two deep reddish-brown homogeneous P-450 fractions (designated P-450$_{soy}$ and P-450$_{soy\Delta}$) with specific contents of 15.0 to 16.8 nmol of P-450 per mg of protein were isolated from crude extracts of *S. griseus*. Results from a typical purification are given in Table 1. The A_{394}/A_{280} ratios of purified preparations were usually between 2.1 and 2.3. The elution profiles of the final hydroxylapatite HPLC step are depicted in Fig. 1.

Molecular weight and isoelectric point determination. The molecular weights of native P-450$_{soy}$ and P-450$_{soy\Delta}$ were estimated to be 48,000 and 46,000, respectively, by size-exclusion HPLC (LKB TSK G3000 SW column [7.5 by 600 mm]). LDS-PAGE analysis of the reduced and denatured enzymes revealed single bands for each protein (Fig. 2), and the molecular weights of P-450$_{soy}$ and P-450$_{soy\Delta}$ were estimated to be 47,500 and 46,000, respectively. The nearly identical molecular weight estimates for both the native and denatured proteins indicate that the native *S. griseus* cytochromes P-450 are monomers.

Both P-450 enzymes migrated as single bands on isoelectric focusing analyses (Pharmacia Phast Gel IEF 3-9; data not shown), with pI values of 5.7 and 6.0 for P-450$_{soy}$ and P-450$_{soy\Delta}$, respectively. These pI values are at least 0.8 pH units more alkaline than those reported for other procaryotic cytochromes P-450 (2, 8, 9, 32).

Spectral characteristics and identification of the heme prosthetic group. The absorption spectra of P-450$_{soy}$ are depicted in Fig. 3. The absolute spectra (measured at pH 6.8 and 23°C) of oxidized (maxima at 394, 514, and 646 nm), reduced (411 and 545 nm), and CO-reduced (447 and 548 nm) P-450$_{soy}$ and P-450$_{soy\Delta}$ were identical. Both enzymes exhibited CO-reduced difference maxima at 448 nm. The spectra of the

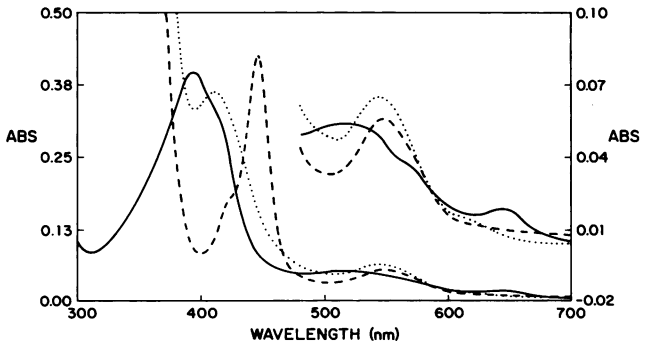

FIG. 3. Absolute absorption spectra of purified P-450$_{soy}$. The concentration of P-450$_{soy}$, based on the heme content, was 4.0 μM in 100 mM sodium phosphate buffer, pH 6.8. The oxidized (——), sodium dithionite-reduced (· · · · · · ·), and CO-reduced complex (---) spectra are shown. ABS, Absorption. Absorption units on the right side are for the spectra from 500 to 700 nm.

TABLE 2. Catalytic activity of cytochrome P-450$_{soy}$

Assay mixture	Product (nmol formed/min per nmol of P-450$_{soy}$)		
	Precocene II		7-Hydroxy-coumarin
	cis-Dihydrodiol	*trans*-Dihydrodiol	
Complete[a]	0.6	2.3	2.7
Minus P-450	<0.1	<0.1	<0.1
Minus ferredoxin	<0.1	<0.1	<0.1
Minus ferredoxin reductase	<0.1	<0.1	<0.1

[a] As described in Materials and Methods.

oxidized enzymes, with the Soret maximum at 394 nm and a weak charge transfer band at 646 nm, are indicative of predominantly high-spin cytochromes P-450.

The spectra of the reduced pyridine hemochrome derivatives of P-450$_{soy}$ and P-450$_{soy\Delta}$ are characteristic of ferroprotoporphyrin IX (10). Based on molecular weights of 47,500 for P-450$_{soy}$ and 46,000 for P-450$_{soy\Delta}$, the molar ratios of heme to protein were calculated as 0.95:1.0 and 0.94:1.0, respectively.

Cytochrome P-450 catalytic activities. The catalytic activities of P-450$_{soy}$ and P-450$_{soy\Delta}$ were investigated with two model substrates, precocene II and 7-ethoxycoumarin, which are both metabolized by intact cells of *S. griseus* following growth on soybean flour-enriched medium (25, 30). In the absence of the indigenous electron transport proteins from the *S. griseus* system, spinach ferredoxin reductase and spinach ferredoxin were used to provide reducing power from NADPH to the terminal cytochrome P-450 component.

TLC analysis of extracts from reconstituted incubations with P-450$_{soy}$ and P-450$_{soy\Delta}$ as enzyme catalysts revealed the formation of the *cis*- and *trans*-precocene II dihydrodiols from precocene II, while 7-ethoxycoumarin was O-dealkylated to 7-hydroxycoumarin (Table 2). The catalytic rates for P-450$_{soy\Delta}$ were within 9% of those determined for P-450$_{soy}$. Removal of any one of the three protein components of the reconstituted cytochrome P-450 system resulted in the loss of catalytic activity.

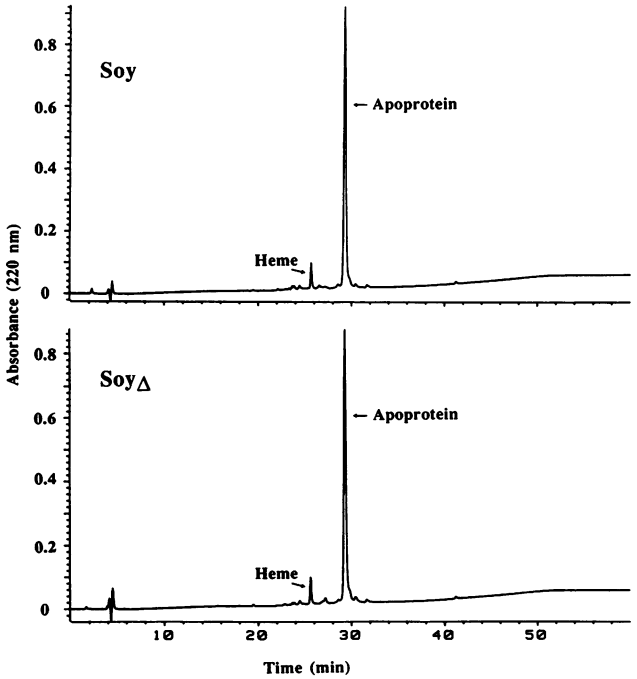

FIG. 4. Elution profiles of 1 nmol each of P-450$_{soy}$ (A) and P-450$_{soy\Delta}$ (B) by RPHPLC.

Cytochrome P-450 apoprotein. Apoproteins of the enzymes were generated in preparation for amino acid analysis and proteolytic mapping of RPHPLC with the procedures outlined in Materials and Methods. RPHPLC removes the heme moiety from the P-450 holoenzyme, resulting in the formation of the apoprotein. The heme group eluted prior to the apoproteins at 19.1 ml. Both P-450$_{soy}$ and P-450$_{soy\Delta}$ showed single protein peaks with identical elution volumes (22.0 ml) (Fig. 4). A mixture of the two proteins was not resolvable when analyzed by this procedure.

Amino acid composition. The amino acid compositions of P-450$_{soy}$ and P-450$_{soy\Delta}$ together with the reported amino acid contents of other procaryotic cytochromes P-450 are shown in Table 3. The amino acid contents of the two *S. griseus* proteins were very similar, with 8 of the 17 amino acids determined having identical residue counts. Like other cytochromes P-450 (4), P-450$_{soy}$ and P-450$_{soy\Delta}$ had an exceptionally high leucine content. The unusually low content of the basic amino acid lysine in P-450$_{soy}$ and P-450$_{soy\Delta}$ is apparently balanced by the conservative substitution of arginine, which could be attributed to the high G+C content in *Streptomyces* DNA and resulting bias toward arginine in codon usage (3).

Western blot analyses. The antigenic relatedness between P-450$_{soy}$ and P-450$_{soy\Delta}$ was assessed by Western blot analyses with polyclonal antisera raised against either P-450$_{soy}$ or P-450$_{soy\Delta}$. Our results show that the two antisera crossreacted with both P-450$_{soy}$ and P-450$_{soy\Delta}$.

TABLE 3. Procaryotic cytochrome P-450 amino acid compositions[a]

Amino acid[b]	No. of residues/mol				
	P-450$_{cam}$	P-450$_{c}$	P-450$_{meg}$	P-450$_{soy}$	P-450$_{soy\Delta}$
Cys[c]	6	2	10	4	4
Glx	55	54	55	43	41
Asx	36	33	49	37	35
Ala	34	54	25	48	47
Ile	24	14	27	14	14
Leu	40	46	44	54	54
Met	9	8	7	6	6
Phe	17	17	17	17	16
Pro	27	25	25	36	36
Trp	1	1	1	ND[d]	ND
Val	24	25	27	25	25
Gly	26	33	27	32	31
Ser	21	20	34	14	14
Thr	19	14	24	24	21
Tyr	9	6	11	5	4
His	12	12	9	14	14
Lys	13	14	28	5	4
Arg	24	29	15	51	49
Total residues	397	407	435	429	415
% Hydrophobic	44	47	40	47	48
% Basic	12	14	12	16	16

[a] Values for P-450$_{cam}$, P-450$_{c}$, and P-450$_{meg}$ are from references 8, 9, and 2, respectively.

[b] Hydrophobic amino acids: Ala, Ile, Leu, Met, Phe, Pro, Trp, and Val. Basic amino acids: His, Lys, and Arg.

[c] Determined as carboxymethylated cysteine.

[d] ND, Not determined.

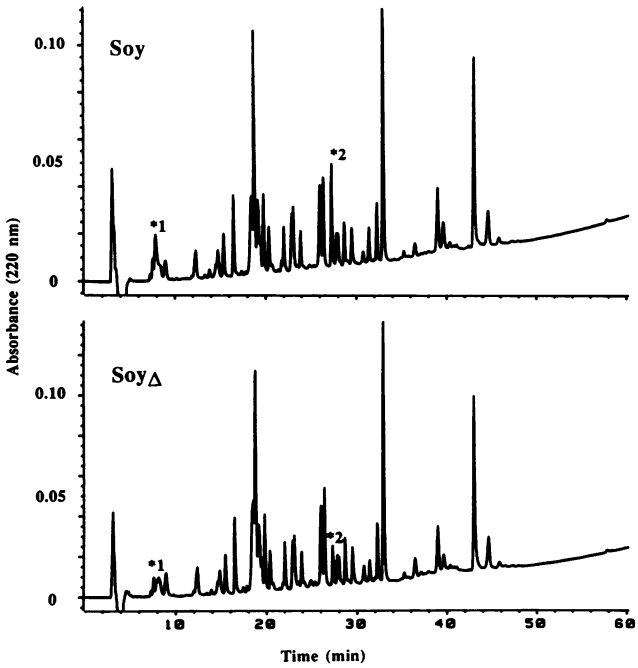

FIG. 5. Tryptic mapping of P-450$_{soy}$ and P-450$_{soy\Delta}$ by RPHPLC. Differences between the two maps are indicated by asterisks.

Proteolytic mapping. To clarify the relationship between P-450$_{soy}$ and P-450$_{soy\Delta}$, we digested these proteins with TPCK-treated trypsin and then mapped the resulting peptides by RPHPLC (Fig. 5). Comparison of the two proteolytic maps provided evidence that P-450$_{soy}$ and P-450$_{soy\Delta}$ were composed of >99% identical peptide fragments. The peak labeled *2 varied in height in different samples and may represent incomplete peptide digestion. The peak labeled *1 probably represents a genuine difference in peptide composition.

The fact that P-450$_{soy}$ and P-450$_{soy\Delta}$ are distinguishable by electrophoretic and chromatographic means while possessing similar antigenicities, amino acid compositions, peptide maps, and enzymatic activities suggests in vitro proteolysis of the larger polypeptide, P-450$_{soy}$, during the purification process. The presence of both P-450$_{soy}$ and P-450$_{soy\Delta}$ in ammonium sulfate extracts (step 2 of the purification scheme) which had or had not been treated with protease inhibitors following cell breakage was investigated by anion-exchange HPLC (note the shallow gradient used, which allows resolution of the two cytochromes by this technique) (Fig. 6A and B) and by Western blot analysis (Fig. 6C and D). It is evident from Fig. 6 that inclusion of the protease inhibitors up to the ammonium sulfate fractionation step inhibited the formation of P-450$_{soy\Delta}$.

Remarkably, subsequent gel filtration chromatography (step 3 of the purification scheme) resulted in the loss of the protection afforded by the protease inhibitors despite prior equilibration of the column and their inclusion in the eluting buffer, which accounts for the presence of P-450$_{soy\Delta}$ and ultimately its isolation with P-450$_{soy}$ in the final purification step.

DISCUSSION

Procedures described in this communication allow the resolution of the P-450 fraction prepared from crude extracts

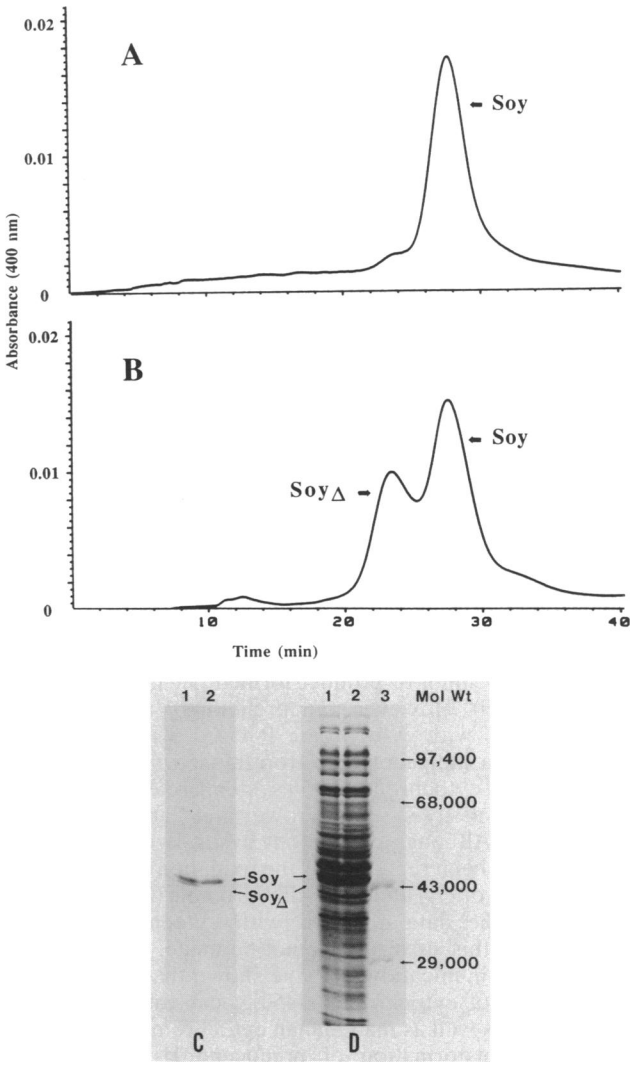

FIG. 6. (A and B) Analysis of ammonium sulfate extracts (2 nmol of P-450) prepared in both the presence and absence of protease inhibitors by anion-exchange HPLC and Western blot analyses. The 390-nm anion-exchange HPLC profiles of the protease-inhibited ammonium sulfate and noninhibited ammonium sulfate preparations are shown in panels A and B, respectively. (C and D) Western blot and LDS-PAGE analyses of the ammonium sulfate extracts of the non-protease-inhibited (lane 1, 100 μg of protein) and inhibited fractions (lane 2, 100 μg of protein) are shown in panels C and D, respectively. The membranes were probed with a 1:2,000 dilution and the anti-P-450$_{soy}$ serum. Lane 3, Molecular weight standards.

of soybean flour-grown *S. griseus* cells into two homogeneous proteins designated P-450$_{soy}$ and P-450$_{soy\Delta}$. The results presented demonstrate, however, that the catalytically active P-450$_{soy\Delta}$ is generated during the purification from P-450$_{soy}$ by in vitro proteolysis.

The purified P-450$_{soy}$ exhibits properties similar to those of previously described cytochromes P-450. P-450$_{soy}$ exists as a single polypeptide with a molecular weight of 47,500. This is within the range (45,000 to 57,000) described for the majority of cytochromes P-450 (1, 2, 4, 21, 22, 32). The spectral characteristics of the P-450$_{soy}$ are also similar to those of the other purified cytochromes P-450 (4), except that the spectrum of the substrate-free ferricytochrome, with a Soret maximum at 394 nm, is characteristic of a high-spin

cytochrome P-450. The majority of the purified eucaryotic cytochromes P-450 and all of their procaryotic counterparts, in the absence of substrate or detergents, are low spin. Cytochrome P-450_{soy} is therefore the first substrate-free high-spin procaryotic P-450 to be reported, ranking among the limited number of high-spin cytochromes P-450, all isolated from either rat liver (24, 31) or rabbit liver (5, 13) sources.

The requirement by cytochrome P-450_{soy} for both ferredoxin reductase and ferredoxin for activity indicates that the *S. griseus* cytochrome P-450 system is similar to other reported procaryotic P-450 systems in being multicomponent. The only described procaryotic cytochrome P-450 that differs from this arrangement is the catalytically self-sufficient P-450_{BM-3}, which contains both reductase and monooxygenase activities on a single 119,000 M_r polypeptide (20). The ability of cytochrome P-450_{soy} to accept reducing equivalents from spinach chloroplast ferredoxin demonstrates that the *S. griseus* enzyme is relatively nonspecific in its interaction with the component providing reducing power, although this step is rate limiting overall. The rates obtained for O-dealkylation of 7-ethoxycoumarin (Table 2) by the P-450_{soy} spinach ferredoxin-coupled system reported in this communication are within the lower range of activities for the same reaction catalyzed by mammalian cytochromes P-450 (0 to 27 nmol of product formed per min per nmol of P-450) (12, 19). However, our preliminary results indicate that activities with cytochrome P-450_{soy} will be enhanced once the other indigenous electron transport components of the *S. griseus* cytochrome P-450 system are employed in the reconstituted assay system (Trower and Sariaslani, unpublished data). Although carried out by rat liver microsomes, no rates have been reported for the oxidation of precocene II to its mixture of *cis*- and *trans*-dihydrodiols (15), so comparisons with the data obtained with cytochrome P-450_{soy} (Table 2) for this substrate cannot be made.

In this communication we have shown that homogeneous preparations of cytochrome P-450_{soy} can mimic *S. griseus* intact cells, as well as mammalian cytochromes P-450, in the oxidative transformation of precocene II and 7-ethoxycoumarin. These findings provide strong evidence for the involvement of cytochrome P-450_{soy} in the in vivo oxidation of these two substrates, confirming the original suggestion by Sariaslani and Kunz (29). The involvement of cytochrome P-450_{soy} in other biotransformation reactions performed by *S. griseus* intact cells grown on soybean flour-enriched medium and the resemblance of this enzyme to its mammalian counterparts will be investigated further in this laboratory.

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LITERATURE CITED

1. **Appleby, C. A.** 1978. Purification of *Rhizobium* cytochromes P-450. Methods Enzymol. **52**:157–166.
2. **Berg, A., M. Ingelman-Sundberg, and J.-Å. Gustafsson.** 1979. Purification and characterization of cytochrome P-450_{meg}. J. Biol. Chem. **254**:5264–5271.
3. **Bibb, M. J.. P. R. Findlay, and M. W. Johnson.** 1984. The relationship between base composition and codon usage in bacterial genes and its use for the simple and reliable identification of protein-coding sequences. Gene **30**:157–166.
4. **Black, S. D., and M. J. Coon.** 1986. Comparative structures of P-450 cytochromes, p. 161–216. *In* P. R. Ortiz de Montellano (ed.), Cytochrome P-450: structure, mechanism and biochemistry. Plenum Press, New York.
5. **Boström, H., and K. Wikvall.** 1982. Hydroxylations in biosynthesis of bile acids. J. Biol. Chem. **257**:11755–11759.
6. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
7. **Chien, M., and J. P. Rosazza.** 1981. Microbial transformations of natural antitumor agents. XV. Metabolism of bruceantin by *Streptomyces griseus*. J. Chem. Soc. Perkin Trans. **I**:1352–1356.
8. **Dus, K., M. Katagiri, C.-A. Yu, D. L. Erbes, and I. C. Gunsalus.** 1970. Chemical characterization of cytochrome P-450_{cam}. Biochem. Biophys. Res. Commun. **40**:1423–1430.
9. **Dus, K., R. Goewert, C. C. Weaver, and D. Carey.** 1976. P-450 hemeproteins of *Rhizobium japonicum*. Biochem. Biophys. Res. Commun. **69**:437–445.
10. **Fuhrhop, J.-H., and K. M. Smith.** 1975. Laboratory methods in porphyrin and metalloporphyrin research, p. 48–55. Elsevier Scientific Publishing Co., New York.
11. **Gornall, A. G., C. J. Bardawill, and M. M. David.** 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. **177**:751–766.
12. **Guengerich, F. P., G. A. Dannan, S. T. Wright, M. V. Martin, and L. S. Kaminsky.** 1982. Purification and characterization of liver microsomal cytochromes P-450: electrophoretic, spectral, catalytic, and immunochemical properties and inducibility of eight isozymes isolated from rats treated with phenobarbital or β-naphthoflavone. Biochemistry **21**:6019–6030.
13. **Haugen, D. A., and M. J. Coon.** 1976. Properties of electrophoretically homogenous phenobarbital-inducible and β-naphthoflavone-inducible forms of liver microsomal P-450. J. Biol. Chem. **251**:7929–7939.
14. **Hawkes, R., E. Niday, and J. Gorden.** 1982. A dot-immunobinding assay for monoclonal and other antibodies. Anal. Biochem. **119**:142–147.
15. **Hsia, M. T. S., S. Grossman, and K. R. Schrankel.** 1981. Hepatotoxicity of the antijuvenile hormone precocene II and the generation of dihydrodiol metabolites. Chem. Biol. Interactions **37**:265–277.
16. **Kotake, A. N., and Y. Funae.** 1980. High performance liquid chromatography technique for resolving multiple forms of hepatic membrane-bound cytochrome P-450. Proc. Natl. Acad. Sci. USA **77**:6473–6475.
17. **Kunz, D. A., G. S. Reddy, and A. Vatvars.** 1985. Identification of transformation products arising from bacterial oxidation of codeine by *Streptomyces griseus*. Appl. Environ. Microbiol. **50**:831–836.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
19. **Morgan, E. T., D. R. Koop, and M. J. Coon.** 1982. Catalytic activity of cytochrome P-450 isozyme 3a isolated from liver microsomes of ethanol treated rabbits. J. Biol. Chem. **257**: 13951–13957.
20. **Narhi, L. O., and A. J. Fulco.** 1986. Characterization of a catalytically self-sufficient 119,000-dalton cytochrome P-450 monooxygenase induced by barbiturates in *Bacillus megaterium*. J. Biol. Chem. **261**:7160–7169.
21. **O'Keefe, D. P., J. A. Romesser, and K. J. Leto.** 1988. Identification of constitutive and herbicide inducible cytochromes P-450 in *Streptomyces griseolus*. Arch. Microbiol. **149**:406–412.
22. **O'Keeffe, D. H., R. E. Ebel, and J. A. Peterson.** 1978. Purification of bacterial cytochrome P-450. Methods Enzymol. **52**: 151–157.
23. **Omura, T., and R. Sato.** 1964. The carbon monoxide binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J. Biol. Chem. **239**:2370–2378.
24. **Patten, C. J., S. M. Ning, A. Y. H. Lu, and C. S. Yang.** 1986. Acetone-inducible cytochrome P-450: purification, catalytic activity, and interaction with cytochrome b_5. Arch. Biochim. Biophys. **251**:629–638.
25. **Sariaslani, F. S., and J. P. Rosazza.** 1983. Novel biotransforma-

tions of 7-ethoxycoumarin by *Streptomyces griseus*. Appl. Environ. Microbiol. **46**:468–474.
26. **Sariaslani, F. S., and J. P. Rosazza.** 1984. Biocatalysis in natural products chemistry. Enzyme Microb. Technol. **6**:242–253.
27. **Sariaslani, F. S., F. M. Eckenrode, J. M. Beale, and J. P. Rosazza.** 1984. Formation of a reactive iminium derivative by enzymatic and chemical oxidations of 16-*O*-acetylvindoline. J. Med. Chem. **27**:749–754.
28. **Sariaslani, F. S., and J. P. Rosazza.** 1985. Biotransformation of 1′,2′-dihydrororotenone by *Streptomyces griseus*. Appl. Environ. Microbiol. **49**:451–452.
29. **Sariaslani, F. S., and D. A. Kunz.** 1986. Induction of cytochrome P-450 in *Streptomyces griseus*. Biochem. Biophys. Res. Commun. **141**:405–410.
30. **Sariaslani, F. S., L. R. McGee, and D. W. Ovenall.** 1987. Microbial transformation of precocene II: oxidative reactions by *Streptomyces griseus*. Appl. Environ. Microbiol. **53**:1780–1784.
31. **Seidel, S. L., and T. K. Shires.** 1986. Purification and characterization of a previously unreported form of cytochrome P-448 from the liver of 3-methylcholanthrene-pretreated rats. Biochem. J. **235**:859–868.
32. **Shafiee, A., and C. R. Hutchinson.** 1987. Macrolide antibiotic biosynthesis: isolation and properties of two forms of 6-deoxyerythronolide B hydroxylase from *Saccharopolyspora erythraea* (*Streptomyces erythreus*). Biochemistry **26**:6204–6210.
33. **Shively, J. E.** 1986. Reverse-phase HPLC isolation and microsequence analysis, p. 41–87. *In* J. E. Shively (ed.), Methods of protein microcharacterization. The Humana Press Inc., Clifton, N.J.
34. **Smith, R. V., and P. J. Davis.** 1980. Induction of xenobiotic monooxygenases. Adv. Biochem. Eng. **2**:61–100.
35. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
36. **Wislocki, P. G., G. T. Miura, and A. Y. H. Lu.** 1980. Reactions catalyzed by the cytochrome P-450 system, p. 135–182. *In* W. B. Jakoby (ed.), Cytochrome P-450. Academic Press, New York.