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Purification and properties of a shortened form of cytochrome P-450 2E1: Deletion of the NH_2-terminal membrane-insertion signal peptide does not alter the catalytic activities

(rabbit liver microsomal cytochrome P-450/membrane binding/alcohol-inducible P-450/heterologous expression in *Escherichia coli*/alcohol oxidation)

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ABSTRACT **As reported previously, alcohol-inducible cytochrome P-450 2E1 lacking the hydrophobic NH_2-terminal segment is located primarily in the inner cell membrane when expressed in *Escherichia coli* and is active with a typical substrate. To study the catalytic properties in detail, we have purified the truncated P-450 lacking residues 3–29 to electrophoretic homogeneity from the solubilized bacterial membrane fraction in the presence of 4-methylpyrazole as a stabilizing agent. The resulting heme protein with a specific content of 15.8 nmol of P-450 per mg of protein has a reduced CO difference spectrum identical to that of the full-length enzyme, with a Soret maximum at 452 nm. The rates of catalysis of four reactions in the reconstituted enzyme system, including the oxygenation of ethanol to give acetaldehyde, the oxidative dealkylation of *N*-nitrosodiethylamine to give ethylene and acetaldehyde, and the ring hydroxylation of aniline and *p*-nitrophenol, are the same with the shortened and full-length enzymes. The apparent K_m of *p*-nitrophenol is also the same, as is that for NADPH-cytochrome P-450 reductase and for cytochrome b_5, which stimulates *p*-nitrocatechol formation about 3-fold. Moreover, the requirement for phosphatidylcholine for full catalytic activity is unchanged despite the absence of the NH_2-terminal segment. Although this highly hydrophobic segment is believed to play a role in the intact cell as a membrane-insertion signal sequence, we conclude that it has no function in the catalytic activity of the cytochrome as an oxygenase, including interactions with the other components of the enzyme system.**

Cytochrome P-450 isoforms are found in an array of organisms from bacteria to man. Many of the enzymes, and particularly those in the hepatic endoplasmic reticulum, are remarkable in their ability to metabolize a wide variety of both physiologically occurring and xenobiotic compounds. Despite large differences in substrate specificity and in amino acid sequence, the P-450s are thought to share many structural motifs, as reviewed (1). However, studies on structure–function relationships have been limited by the lack of a three-dimensional structure for a mammalian isoform. Most models for mammalian P-450s are based on the known structure of P-450_{cam} (2), a cytochrome from *Pseudomonas putida* that exhibits less than 20% sequence identity with the mammalian isoforms. P-450_{cam} is a cytosolic protein, whereas mammalian P-450s, whether found in the mitochondria or endoplasmic reticulum, are integral membrane proteins. The microsomal isoforms are inserted into the membrane of the endoplasmic reticulum in a signal-recognition-particle-dependent manner in which the NH_2-terminal segment acts as a noncleavable signal sequence (3). It is generally accepted that this signal peptide serves as either all or part of the anchor to the membrane. Thus, the two currently accepted possibilities for P-450 membrane topology are a P-450_{cam}-like cytosolic domain anchored by either a single NH_2-terminal segment or by a hairpin loop composed of this segment and the following hydrophobic segment (4). The subcellular location of the NH_2-terminal residue, which would differentiate between these possibilities, has not been resolved. Bernhardt *et al.* (5) concluded that the orientation was cytosolic in experiments in which the NH_2 terminus of P-450 in hepatic microsomes was labeled with fluorescein isothiocyanate, whereas Vergères *et al.* (6) reported that the orientation was luminal as a result of studies in which P-450 2B1 could not be labeled after reconstitution into liposomes.

If the single transmembrane segment model is correct, a microsomal P-450 with the NH_2-terminal segment removed might have a structure closely resembling that of bacterial P-450_{cam}, a cytosolic protein. We have addressed this possibility with the use of a bacterial expression system for P-450 2E1 (7). Unexpectedly, the truncated enzyme lacking this hydrophobic segment was bound to the bacterial inner membrane (8). In the present study, we describe the purification of the shortened enzyme expressed in *Escherichia coli* and show that it retains full catalytic properties. Moreover, the requirement for phospholipid for full activity is the same as that of the intact cytochrome (9). The interactions of the shortened P-450 with cytochrome P-450 reductase and cytochrome b_5 are also apparently unchanged, arguing that this NH_2-terminal segment is not essential to catalytic function.

MATERIALS AND METHODS

Cell Culture. The expression vector and cDNA used were as described (7, 8). *E. coli* MV1304 was grown in a 200-liter fermentor as follows: 180 liters of Luria–Bertani medium was inoculated with a 2-liter overnight culture, and cells were grown at 37°C with high aeration to an OD_{600} value of 0.5–0.8, at which time 4-methylpyrazole, a high-affinity ligand for P-450 2E1 (10), was added to 5 μM (final concentration), and synthesis of the P-450 2E1 was induced by the addition of isopropyl thiogalactoside at a final concentration of 1.0 mM. After 4 hr the cells were harvested with a Sharples centrifuge and stored frozen at −70°C.

Membrane Isolation. Frozen cells were suspended in 20 mM potassium phosphate (pH 7.4) containing 50 mM KCl, 5 mM EDTA, and 50 μM 4-methylpyrazole, at approximately 1 g of cells per 3 ml of buffer. The cells were lysed by three passes through a Manton–Gaulin homogenizer at 900 psi, and cell debris was removed by centrifugation at 10,000 × *g* for

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Table 1. Purification of shortened P-450 2E1 from *E. coli* membranes

Preparation	Protein, mg	P-450, nmol	Yield, %	P-450 content, nmol/mg of protein
Membrane fraction*	2770			
S-Sepharose eluate	280	65	100	0.23
Hydroxyapatite eluate		61	94	
DEAE-Sepharose eluate		45	69	
Hydroxyapatite eluate	2.0	32	49	15.8

*P-450 could not be determined spectrally in the membrane fraction.

15 min. The membrane fraction was sedimented by ultracentrifugation at 142,000 × *g* for 1 hr at 4°C, resuspended in the starting volume of 50 mM potassium phosphate (pH 7.4) containing 0.8 M KCl, 1.0 mM EDTA, and 50 μM 4-methylpyrazole, pelleted, and then suspended and homogenized in about half the starting volume of 10 mM potassium phosphate (pH 6.4) containing 1.0 mM EDTA, 20% (vol/vol) glycerol, and 50 μM 4-methylpyrazole.

Purification of Shortened P-450 2E1. The procedure is summarized in Table 1. All buffers contained 20% glycerol and 50 μM 4-methylpyrazole. The membrane fraction (at a concentration of 5 mg of protein per ml) was solubilized by the addition of 12.5% (vol/vol) aqueous *n*-octyl glucoside (Sigma) to give a final concentration of 1.25%, with continuous mixing at room temperature. After 20 min the mixture was centrifuged at 142,000 × *g* for 45 min at 4°C, and the supernatant layer was loaded onto an S-Sepharose column (30 mg of protein per ml of resin) previously equilibrated with homogenization buffer containing 1% octyl glucoside. The column was washed with 5 column volumes of 100 mM potassium phosphate (pH 6.4) containing 1.0 mM EDTA and 1% octyl glucoside, and the cytochrome was then eluted with 150 mM potassium phosphate (pH 6.6) containing 1.0 mM EDTA and 1% octyl glucoside. Fractions containing P-450 were pooled and diluted with 4 volumes of 10 mM potassium phosphate (pH 7.7). The P-450 concentration of the sample was determined from the ferrous carbonyl difference spectrum with a coefficient of 80 mM^{-1} for the absorbance difference between 452 and 490 nm (11). Further purification was as follows: the diluted sample was loaded onto a column of hydroxyapatite (Bio-Rad) (10 nmol of P-450 per ml of column volume) equilibrated with 10 mM potassium phosphate (pH 7.7) containing 0.2% Tergitol type NP-10. The column was washed with 2 column volumes of this buffer and then with 2 column volumes of 30 mM potassium phosphate (pH 7.7) containing 0.2% Tergitol NP-10. The P-450 was eluted with 400 mM potassium phosphate (pH 7.7) containing 0.2% Tergitol NP-10. Fractions containing P-450 were pooled, concentrated, and dialyzed with the use of a ProDiCon apparatus (Bio-Molecular Dynamics, Beaverton, OR) against 10 mM potassium phosphate (pH 7.7) containing 0.1 mM EDTA and 0.2% Tergitol NP-10. The concentrated sample was diluted with 3 volumes of 0.5% Tergitol NP-10 and then loaded onto a DEAE-Sepharose column (10 nmol of P-450 per ml of resin) equilibrated with 3.0 mM potassium phosphate (pH 7.7) containing 0.1 mM EDTA and 0.5% Tergitol NP-10. The column was washed with 2 column volumes of the equilibration buffer, and the P-450 was eluted with a 15-column-volume gradient from 10 to 100 mM potassium phosphate (pH 7.7) containing 0.1 mM EDTA and 0.5% Tergitol NP-10. Those fractions containing only the P-450, as determined by SDS/PAGE, were pooled and loaded onto a hydroxyapatite column (20 nmol of P-450 per ml of column volume) to remove the detergent. The column was washed with 10 mM potassium phosphate (pH 7.4) until the absorbance of the eluate at 276 nm was less than 0.01. The enzyme was eluted with 0.5 M potassium phosphate (pH 7.4) containing 0.1% sodium cholate and then dialyzed against 100 mM potassium phosphate (pH 7.4) containing 1.0 mM EDTA and 20 μM 4-methylpyrazole. The final preparation had a specific content of 15.8 nmol of P-450 per mg of protein.

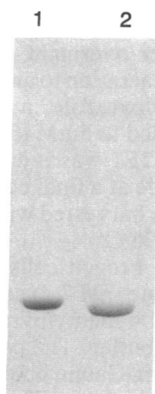

FIG. 1. SDS/polyacrylamide gel electrophoresis of purified P-450 2E1. Lanes: 1, P-450 2E1 isolated from rabbit liver microsomes; 2, shortened P-450 2E1 isolated from *E. coli*. The protein (0.1 μg) was loaded onto a 7.5% gel.

Catalytic Activities. Unless otherwise indicated, all reaction mixtures contained 0.05 μM P-450, 0.15 μM NADPH-cytochrome P-450 reductase, 1,2-dilauroylglycero(3)-phosphocholine (30 μg/ml), and 1.0 mM NADPH in a total volume of 1.0 ml. The formation of acetaldehyde from ethanol was measured by gas chromatography of the headspace gas of a reaction mixture containing 50 mM potassium phosphate (pH 7.6) and 80 mM ethanol, as described by Koop *et al.* (11). Reactions were terminated after a 20-min incubation at 37°C by the addition of 0.2 ml of 30% (vol/vol) perchloric acid. The N-deethylation of *N*-nitrosodiethylamine was determined by the formation of ethylene and acetaldehyde in a reaction mixture containing 50 mM potassium phosphate (pH 7.4), 100 μM substrate, and 2.0 mM NADPH, as described by Ding and Coon (12). Reactions were terminated after a 30-min incubation at 37°C by the addition of 0.2 ml of 30% perchloric acid. The sum of the two products is reported. Aniline *p*-hydroxylation was determined in a reaction mixture containing 50 mM potassium phosphate (pH 7.6) with 2.5 mM substrate and 0.25 μM reductase. Reactions were terminated after incubation at 30°C for 10 min by the addition of 0.3 ml of 20% (wt/vol) trichloroacetic acid, and *p*-aminophenol was measured by the method of Schenkman *et al.* (13). The formation of 4-nitrocatechol from *p*-nitrophenol was determined spectrally as described by Koop (14), with reactions carried out in 100 mM potassium phosphate (pH 6.8) containing 1.0 mM ascorbic acid, 100 μM *p*-nitrophenol, and, where indicated, 0.1 or 0.2

Table 2. Catalytic activities of shortened and full-length forms of P-450 2E1

Substrate	Activity, nmol per min per nmol of P-450	
	Shortened	Full length
Ethanol	35.1 ± 1.0	35.2 ± 2.0
N-Nitrosodiethylamine	4.7 ± 0.2	4.9 ± 0.2
Aniline	21.4 ± 1.0	19.8 ± 1.0
p-Nitrophenol	6.9 ± 0.4	6.7 ± 0.3
p-Nitrophenol + 0.1 nmol of b_5	20.0 ± 0.7	22.0 ± 1.0
p-Nitrophenol + 0.2 nmol of b_5	23.0 ± 1.5	25.4 ± 1.2

Activity values are an average of four to six determinations, presented as mean ± SD.

FIG. 2. NH_2-terminal amino acid sequence of shortened P-450 2E1. The first 10 amino acids were determined by automated Edman degradation, and the others were predicted from the cDNA sequence, which was confirmed by direct sequencing according to the method of Sanger *et al.* (17). Hydrophobic amino acids (•) and charged residues are indicated.

μM cytochrome b_5. Reactions were terminated after a 10-min incubation at 37°C by the addition of 0.5 ml of 35% perchloric acid.

Other Methods. Protein concentrations were determined by the bicinchoninic acid method (Pierce) with bovine serum albumin as standard. NADPH-cytochrome P-450 reductase and cytochrome b_5 were prepared from rabbit liver microsomes by the methods of French and Coon (15) and Strittmatter *et al.* (16), respectively. SDS/PAGE and staining with silver nitrate were as described (8).

RESULTS AND DISCUSSION

Table 1 summarizes the purification of shortened P-450 2E1 from *E. coli*. The concentration of P-450 in the starting membrane fraction could not be determined accurately due to the relatively high concentration of bacterial cytochromes *o* and *d*, which have reduced CO difference spectra that interfere with the quantitation of P-450 (8). Approximately 50% final yield is obtained based on the eluate from the S-Sepharose column, at which stage the P-450 level can be measured accurately. Apparently all of the cytochrome *d* and much of the cytochrome *o* in the preparation are removed by column chromatography on S-Sepharose, as neither binds well under the mildly acidic conditions used. The major contaminant after the S-Sepharose chromatography is residual cytochrome *o*, which is separated from the P-450 on the DEAE-Sepharose column. SDS/PAGE (Fig. 1) showed that the final preparation (lane 2) is homogeneous and runs slightly ahead of the full-length P-450 2E1 isolated from rabbit liver microsomes (lane 1).

Purified shortened P-450 has spectral characteristics identical to those of P-450 2E1 isolated from rabbit liver, with a reduced CO absorbance maximum at 452 nm that is characteristic of this cytochrome (data not shown). The absolute spectrum of the ferric cytochrome exhibits a maximum at 424 nm due to the presence of the ligand 4-methylpyrazole, which is added to maintain the active form of the enzyme (8). In the absence of 4-methylpyrazole, the shortened cytochrome has very low catalytic activity; the inclusion of this high-affinity ligand in the solubilization and purification buffers is necessary to obtain an enzyme that is fully active, as compared to the full-length cytochrome (see below). These observations indicate that the removal of the hydrophobic NH_2-terminal segment somehow destabilizes the enzyme but that addition of the high-affinity ligand prevents the loss of activity of the truncated cytochrome. Moreover, we have found that the addition of 4-methylpyrazole to the cell culture at the time of induction results in an increase in the amount of immunodetectable protein in whole-cell lysates, suggesting that the ligand also stabilizes the enzyme *in vivo*.

Fig. 2 shows the NH_2-terminal sequence of the shortened protein, as determined for the first 10 residues by automated Edman degradation. The sequence is as expected from the cDNA sequence (8), except that the predicted NH_2-terminal methionine is not present, as is also the case with P-450 2E1 isolated from rabbit liver microsomes. It is worth noting that the sequence is still significantly hydrophobic, with no charged amino acids in the first 20 positions. The shortened form remains tightly bound to the bacterial inner membrane, as indicated by our inability to dissociate it with a 1.0 M KCl or 0.1 M Na_2CO_3 wash (8); the lack of charged residues in this initial segment may allow it to insert into the membrane and serve as an anchor for the shortened P-450. However, it is not yet known whether, in the full-length enzyme, this same (second) hydrophobic segment is also integrated into the membrane and, in particular, whether it is involved in binding the full-length cytochrome to the endoplasmic reticulum of the mammalian cell. Antibody studies (18) and proteolysis experiments (6) suggest that this second segment is extrinsic to the membrane. It should be noted that others have found that similarly truncated P-450s, rat 1A1 expressed in yeast (19), and bovine 17α expressed in COS-1 cells (20) are integrated into the endoplasmic reticulum of the cells, but activity was low in the former case and absent in the latter.

The catalytic activity of the shortened P-450 was examined toward four substrates, which were at saturating levels, and compared to that of P-450 2E1 isolated from rabbit liver microsomes. The substrates selected included ethanol, which is oxygenated to form acetaldehyde, *N*-nitrosodiethylamine, a procarcinogen that is activated by oxidative dealkylation, and aniline and *p*-nitrophenol, both of which undergo hydroxylation on the aromatic ring. The turnover numbers are listed in Table 2. No significant differences were seen between the shortened and full-length enzymes. Further analysis revealed that deletion of the hydrophobic NH_2-terminal

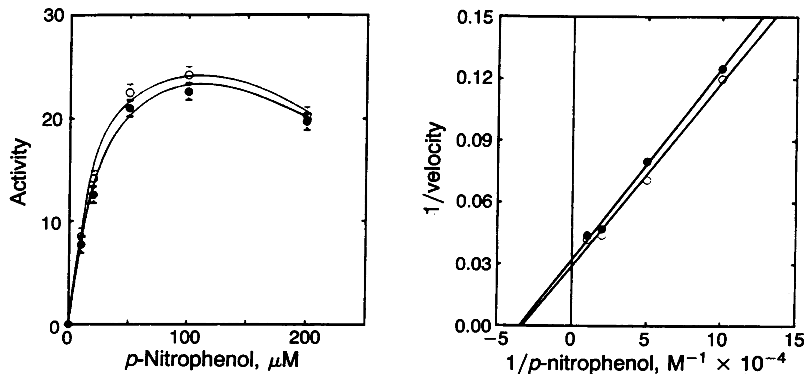

FIG. 3. (*Left*) Effect of substrate concentration on activity of shortened and full-length P-450 2E1. (*Right*) The kinetic constants derived from the Lineweaver–Burke plot are presented in Table 3. Cytochrome b_5 (0.2 μM) was included in all reaction mixtures to increase the activity at low substrate concentrations. Solid circles, shortened P-450 2E1; open circles, P-450 2E1 isolated from rabbit liver microsomes. Activity is reported as nmol per min per nmol of P-450.

Table 3. Kinetic parameters for shortened and full-length P-450 2E1

Reaction component varied	Apparent K_m, μM		V_{max}, nmol per min per nmol of P-450	
	Shortened	Full length	Shortened	Full length
p-Nitrophenol	40.4 ± 1.5	38.0 ± 1.5	27.1 ± 2.0	30.3 ± 2.0
Cytochrome b_5	0.04 ± 0.01	0.03 ± 0.01	28.6 ± 2.0	29.2 ± 1.0
Reductase	0.08 ± 0.01	0.05 ± 0.01	10.3 ± 1.0	8.8 ± 0.5

Values given are the average of two determinations using Lineweaver–Burke plots for substrate hydroxylation (from Fig. 3) and four determinations using Hanes–Woolf plots for reductase and cytochrome b_5 interactions (from Fig. 4). Cytochrome b_5 was included in the reaction mixtures for determination of the kinetic constants of *p*-nitrophenol (as indicated in the legend to Fig. 3) but was omitted for determination of the reductase constants.

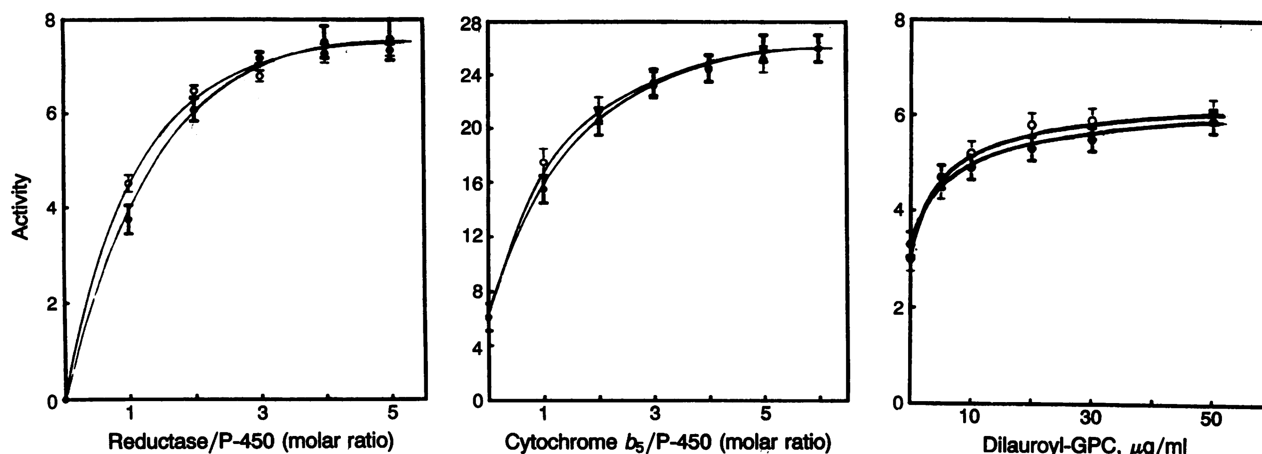

FIG. 4. Effect of reductase, cytochrome b_5, and phospholipid concentration on activity with *p*-nitrophenol as the substrate. Solid circles, shortened P-450 2E1; open circles, P-450 2E1 isolated from rabbit liver. Activity is expressed as nmol per min per nmol of P-450. Dilauroyl-GPC, 1,2-dilauroylglycero(3)phosphocholine.

segment did not change either the apparent K_m or the V_{max} of *p*-nitrophenol (Fig. 3 and Table 3). Saturation plots for the interaction of the shortened and full-length enzymes with NADPH-cytochrome P-450 reductase, cytochrome b_5, and phospholipid are shown in Fig. 4, and the derived kinetic constants for cytochrome b_5 and the reductase are given in Table 3. The slight differences in the apparent K_m values of cytochrome b_5 and NADPH-cytochrome P-450 reductase with the two P-450 preparations are within the range of experimental error for such measurements. Similarly, no differences were seen in the stimulation by 1,2-dilauroylglycero(3)phosphocholine with the shortened and full-length enzymes, indicating that the requirement for phospholipid for full activity is not changed upon deletion of the highly lipophilic NH_2-terminal segment. The increase in activity with phospholipid, first noted with the reconstituted liver microsomal P-450 system (21, 22), has apparently not been observed with the cytosolic bacterial P-450_{cam}. Several groups (23–26) have shown that complex formation between cytochrome P-450 and the reductase is facilitated by phospholipid. Our present results demonstrate that the NH_2-terminal segment of P-450 is not involved in the phospholipid-mediated interaction of these proteins. On the other hand, Lu *et al.* (22), Coon *et al.* (23), Black *et al.* (27), and Gum and Strobel (28) have demonstrated that the hydrophobic NH_2-terminal segment of NADPH-cytochrome P-450 reductase is essential for coupling with cytochrome P-450. It should be noted that electrostatic charge coupling is also important to the interaction of these two enzymes (29–32). Although the NH_2 terminus is believed to play a role *in vivo* as a membrane-insertion signal sequence, our results demonstrate that this segment has no function in the catalytic activity of P-450 2E1 as an oxygenase or even indirectly in the interaction of this P-450 with the reductase or cytochrome b_5.

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