Purification and Characterization of Cytochrome P450 2E2 from Hepatic Microsomes of Neonatal Rabbits[1]

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The alcohol-inducible P450 2E subfamily in the rabbit has two known members that differ in only 16 amino acid residues scattered throughout the polypeptide chain. P450 2E1 has been thoroughly characterized, and is known to have diverse inducers and substrates. Little is known, however, about the properties of P450 2E2, since efforts to isolate this isozyme from adult rabbits have been unsuccessful. In the present study, 2E2 was purified to electrophoretic homogeneity from liver microsomes of neonatal rabbits with the use of 4-methylpyrazole as a stabilizing agent. The purified cytochrome was identified as 2E2 by NH$_2$-terminal amino acid sequence analysis as well as by immunoblot analysis with three different antibodies to 2E1. Purified 2E2, in contrast to 2E1, is predominantly low-spin in the presence of 20% glycerol, but is in a mixed high- and low-spin state as the concentration of glycerol is decreased. The catalytic properties of purified 2E1 and 2E2 were compared in the reconstituted system with a variety of substrates, including alcohols, ethers, nitrosamines, and aromatic compounds. Differences between the two enzymes in catalytic activity and in the interaction with cytochrome b$_5$ were observed with some but not all of the substrates tested. Purified 2E1 and 2E2 both consume molecular oxygen relatively rapidly during NADPH oxidation in the absence of an added substrate, and stoichiometric determinations indicated that only about 20% of the O$_2$ was reduced to H$_2$O$_2$, with the remainder apparently undergoing four-electron reduction to water. © 1991 Academic Press, Inc.

Chronic ethanol consumption by animals and humans has been linked by epidemiological evidence to cancer, chemical toxicities, and other pathological states, with increasing attention being paid in recent years to the role of alcohol-inducible cytochrome P450 (1). In the rabbit, the P450 2E subfamily [see Ref. (2) for recommended nomenclature] has two known members, 2E1[3] and 2E2 (3), which differ in only 16 amino acid residues scattered throughout the polypeptide chain. 2E1 (initially called P450 3a), which was originally purified from liver microsomes of ethanol-treated rabbits in this laboratory (4), has relatively high activity in the oxidation of ethanol and other alcohols (5) as well as of many other compounds of pharmacological and toxicological concern, including nitrosamines, alkanes, halogenated alkanes, ethers, and aromatic compounds (1, 6). This isozyme is also active toward compounds of physiological importance, such as lipid hydroperoxides (7) and acetone (8). Furthermore, purified 2E1 is unusual in that it rapidly consumes O$_2$ and NADPH in the absence of a substrate (9); the activated oxygen species generated by 2E1 have been implicated in pathological processes that may result in tissue damage (10, 11).

Evidence for 2E2 was first obtained by isolation of the gene (3), and subsequent studies showed that 2E2 mRNA in adult rabbit liver is present at half the level of the 2E1 transcript (12). Little is known, however, about the catalytic properties of 2E2 since earlier attempts in our laboratory to isolate the 2E2 protein were unsuccessful. However, a 2E2 cDNA was recently isolated from a rabbit liver cDNA library and expressed in *Saccharomyces cerevisiae* (13). The expressed microsomal 2E2 was found to be active toward *N,N*-diethylnitrosamine and butanol, but the cytochrome was converted to inactive P420 under conditions routinely used for the isolation of 2E1 from adult rabbits. Interestingly, the inactivation was prevented in the presence of 4-MP, a compound known to bind to rabbit 2E1 with particularly high affinity (14).

Recently, Bonfils *et al.* (15) identified a P450 in neonatal rabbit liver (called P450 2y) that was related to, but dis-

[3] Abbreviations used: 2E1 and 2E2, P450 cytochromes 2E1 and 2E2, respectively; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PEG, polyethylene glycol; 4-MP, 4-methylpyrazole; b$_5$, cytochrome b$_5$; NP-10, Tergitol NP-10.

tinct from, 2E1. NH_2-terminal sequence analysis and immunological cross-reaction with anti-2E1 antibody indicated that this neonatal cytochrome was probably 2E2. Their important finding that P450 2y, but not 2E1, was abundant in the hepatic microsomes of 1- to 3-week-old rabbits (15) is in accord with our recent observation that 2E2 mRNA is detectable in rabbit liver immediately after birth whereas 2E1 mRNA is not found until Day 14 (16). The purification of P450 2y from neonatal hepatic microsomes resulted in a preparation of low specific content due, in part, to the presence of a significant amount of P420, and information on the catalytic properties of the partially purified P450 2y was not reported (15).

In the present study, 2E2 was purified from the liver of 2-week-old rabbits to a content of 15.9 nmol of heme/mg of protein with 4-MP present to stabilize the cytochrome during purification, and in other experiments it was copurified with 2E1 from liver microsomes of acetone-treated adult rabbits. We now report the unique spectral properties of 2E2 as well as its catalytic activities, in comparison with those of 2E1, toward a number of substrates in a reconstituted system.

MATERIALS AND METHODS

Animals and microsomes. Two-week-old male New Zealand White rabbits obtained from a local supplier were sacrificed upon arrival. Adult (2.5-kg) male rabbits were treated with 1% acetone in the drinking water for 7 days and allowed free access to Purina rabbit chow. Pooled livers from 20 neonates (approximately 8 g/rabbit) or from 9 acetone-treated adult animals (about 70 g/rabbit) were homogenized, and pyrophosphate-washed microsomes were prepared from the two groups according to the procedure described previously (17) except that 0.05 mM 4-MP was included in all buffers.

Purification of 2E2 from hepatic microsomes of neonatal rabbits. The following protocol is based, with major modification, on the procedure described for the purification of 2E1 from ethanol-treated rabbits (4, 18). Unless otherwise indicated, all buffers contained 0.05 mM 4-MP and 20% glycerol (v/v). Phosphate buffers were prepared as the potassium salt; the fractionation procedures and pH measurements were conducted at 4°C.

In a typical procedure, microsomes from 16 rabbits were suspended in 0.1 M Tris–acetate buffer, pH 7.4, containing 0.1 M KCl and 1 mM EDTA, at a protein concentration of 1.6 mg/ml. NP-10 and sodium cholate were then added as 10% (w/v) solutions to final concentrations of 1% each. The mixture was stirred for 2 h and fractionated with PEG 8000 as described earlier (17). The 7 to 14% PEG supernatant fraction was combined with 2 vol of 10% glycerol (v/v) and applied to a Bio-Rad HTP column (2.5 × 20 cm) previously equilibrated with 5 mM phosphate buffer, pH 7.4, containing 0.2% NP-10 (buffer A). The column was washed with 5 vol of buffer A at 10 mM phosphate, and eluted with a 200-ml linear gradient from 10 to 70 mM phosphate in buffer A and an additional 250 ml of 70 mM phosphate in buffer A. Fractions eluting at 70 mM phosphate that contained 2E2, as judged by SDS-PAGE, were pooled and concentrated to about 50 ml by ultrafiltration with an Amicon PM-30 membrane, dialyzed overnight against two changes (1 liter each) of 3 mM phosphate buffer, pH 7.7, containing 0.6% NP-10 and 0.1 mM EDTA (buffer B), diluted with 2 vol of buffer B, and applied to a DEAE–Sepharose (Pharmacia) column (1 × 20 cm) previously equilibrated with buffer B. The column was washed sequentially with 3 vol of buffer B and 5 vol of buffer B at 20 mM phosphate, and 2E2 was eluted with 10 vol of buffer B at 50 mM phosphate. Samples of column eluate were analyzed by SDS-PAGE, and the fractions with 2E2 as the predominant protein were combined. The pooled fractions, which had a maximal

absorbance at 415 nm and contained b_5 and P450 2C3 (form 3b) in addition to 2E2, were concentrated to less than 40 ml by ultrafiltration, dialyzed against two changes (1 liter each) of 10 mM phosphate buffer, pH 6.4, containing 0.5% NP-10 and 1 mM EDTA (buffer C) for 36 h, and applied to an S-Sepharose column (1 × 20 cm) previously equilibrated with buffer C. The S-Sepharose column was treated sequentially with 5 vol of buffer C (to remove b_5) and a linear gradient (15 vol) of 10 to 200 mM phosphate, pH 6.4, in buffer C. P450 2C3 (maximal absorbance at 417 nm) was eluted at approximately 75 mM phosphate, and 2E2 (with an absorbance maximum at about 422 nm because of 4-MP binding) was eluted in the second half of the gradient and in fractions obtained after further treatment of the column with 200 mM phosphate in buffer C. The pooled 2E2-rich fractions, to which 1/10 vol of 1 M K_2HPO_4 was added to raise the pH, were then dialyzed for 24 h against three changes (1 liter each) of 10 mM phosphate buffer, pH 7.7, containing 0.6% NP-10 and 0.1 mM EDTA (buffer D), and then, after dilution (1:1) with a solution containing 0.6% NP-10 and 20% glycerol, were applied to a second DEAE–Sepharose column (0.7 × 25 cm) previously equilibrated with buffer D. The nonbound fraction and fractions obtained by washing this column with buffer D (10 to 15 vol) had an absorbance maximum at about 424 nm and exhibited a single major band upon SDS-PAGE analysis. These fractions were pooled and, after 1:1 dilution with 5 mM phosphate buffer, pH 7.4 (buffer E), were applied to a second HTP column (0.7 × 6 cm) previously equilibrated with buffer E. The column was washed sequentially with 5 vol each of 10 mM phosphate, 30 mM phosphate, and 45 mM phosphate buffer, pH 7.4, containing 0.2% NP-10, and then with a sufficient amount of 10 mM phosphate in buffer E so that the absorbance of the eluate at 276 nm was less than 0.01. The column was then washed with 3 vol of 10 mM phosphate buffer, pH 7.4, without 4-MP, and the enzyme was eluted with 500 mM phosphate buffer, pH 7.4, containing 0.1% sodium cholate (without 4-MP). The preparation was dialyzed against four changes (100 vol each) of 100 mM phosphate buffer containing 5% glycerol and 0.1 mM EDTA for 24 h to remove cholate and residual 4-MP. The final preparation of 2E2, which was electrophoretically homogeneous and had a content of 15.9 nmol of heme/mg of protein, was stable for at least 2 months when stored at −20°C.
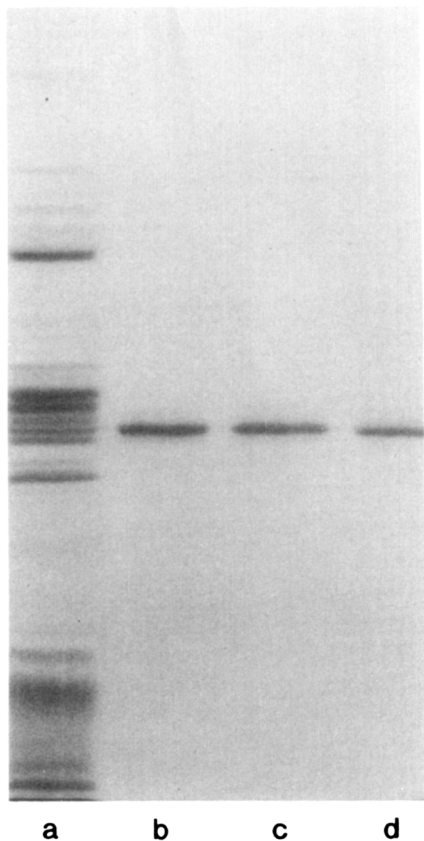
Purification of 2E1 and 2E2 from hepatic microsomes of acetone-treated adult rabbits. Cytochromes 2E1 and 2E2 were copurified from adult liver microsomes in the presence of 4-MP according to essentially the same procedure as described above for the purification of neonatal 2E2, except that the fraction precipitating from 7 to 14% PEG was used and the first HTP column was omitted. The PEG fraction was dialyzed overnight against 200 vol of buffer B and applied to the first DEAE–Sepharose column. The final preparations showed a single band upon SDS–PAGE and had contents of about 18 nmol of heme/mg protein.

Stoichiometric analyses. Stoichiometric determinations were conducted at 30°C under end-point conditions as described by Gorsky *et al.* (9) except that reaction mixtures contained 0.3 μM 2E1 or 2E2, 0.3 μM NADPH–cytochrome P450 reductase, and 30 μg/ml dilauroylglyceryl-3-phosphorylcholine in 100 mM potassium phosphate buffer, pH 7.4. NADPH (150 nmol per milliliter, final concentration) was added to initiate oxygen consumption, which was measured with a Clark-type electrode in conjunction with a Yellow Springs Instrument 35 monitor. The extent of NADPH oxidation was ascertained from the absorbance of the reaction mixture at 340 nm following cessation of oxygen consumption. Aliquots were then removed for measurement of H_2O_2 by the ferrithiocyanate method (19). The initial oxygen concentration was 241 μM as determined by the phenazine methosulfate/NADH reaction (20), and H_2O_2 standard solutions were calibrated by potassium permanganate titration (21).

Monooxygenase assays. The composition of individual reaction mixtures is given in the table legends. All reactions were initiated by the addition of NADPH and carried out at 37°C for lengths of time that represented the initial linear rate of product formation. For reactions in which b_5 was included, the cytochrome was added to the reaction mixture after the addition of the reconstituted P450 system, as described previously (22). The rates of product formation are the average of duplicate or triplicate incubations corrected for zero-time blanks that were

quenched prior to the addition of NADPH. The hydroxylation of *p*-nitrophenol to 4-nitrocatechol was determined as described by Koop (23). The formaldehyde formed from the N-demethylation of *N*-nitrosodimethylamine, *N*-nitroso-*N*-methyl-*N*-phenylamine, and hexamethylphosphoramide, and the O-demethylation of anisole and ethylene glycol dimethyl ether, was measured by the method of Nash (24). The formation of acetaldehyde from ethanol and *N*-nitrosodiethylamine and the formation of butyraldehyde from butanol were determined by gas chromatography of the headspace gas of the reaction mixture as described previously (25, 26). The hydroxylation of aniline to *p*-aminophenol was assayed colorimetrically (27).

Other methods and materials. The concentration of P450 in microsomal suspensions and in crude fractions was determined from the CO difference spectrum of the reduced cytochrome (28). Heme was determined as the reduced pyridine hemochrome (29). The optical spectra of purified 2E1 and 2E2 were determined at 23–25°C with a Cary 219 spectrophotometer. NH$_2$-terminal amino acid sequences were determined by Edman degradation with an Applied Biosystems Model 470A gas-phase protein sequencer (30) at the University of Michigan Protein Sequencing Facility. SDS–PAGE and immunoblot analyses were carried out as previously described (31). Protein was measured routinely by the method of Lowry *et al.* (32) with bovine serum albumin as a standard. The protein content of purified 2E1 and 2E2 was determined by amino acid analysis (33), which gave values 16% less than the Lowry method. The method of Strittmatter *et al.* (34) was used for the purification of

TABLE I

Purification of 2E2 from Hepatic Microsomes of Neonatal Rabbits

Preparation	Protein (mg)	P450 content[a] (nmol/mg protein)	Yield (%)
Pyrophosphate-treated microsomes	1100	0.7	100
Polyethylene glycol (14%) supernatant fraction	471	1.1	67
First HTP column eluate	142	1.2	22
First DEAE-Sepharose column eluate	21.4	3.3	9.2
S-Sepharose column eluate	4.5	9.1	5.3
Second DEAE-Sepharose column eluate	2.6	11.4	3.8
Second HTP column eluate	1.3	15.9	2.7

[a] The P450 concentration for the purified 2E2 is based on the heme content; all other values are based on the determination of P450 as the CO complex. Typical results are presented.

b_5 from rabbit liver microsomes. The electrophoretically homogeneous preparations had specific contents of 40 to 50 nmol of b_5/mg of protein. NADPH–cytochrome P-450 reductase was purified from microsomes obtained from phenobarbital-treated rabbits (35) and had a specific activity of 45 to 60 μmol of cytochrome c reduced per minute per milligram of protein. The preparation and characterization of polyclonal (25) and monoclonal (31) antibodies to rabbit 2E1 have been described. Monoclonal antibody (1-98-1) to rat 2E1 (36) was a gift from Dr. Harry V. Gelboin, National Cancer Institute, NIH; 4-MP was obtained from Sigma; cholic acid, from Aldrich, was recrystallized from 95% ethanol. Other materials were obtained as described previously (4, 25, 31, 37).

RESULTS AND DISCUSSION

Purification of 2E2. Several bands were observed in the molecular weight range expected for P450 enzymes (Fig. 1) when hepatic microsomes from 2-week-old rabbits were submitted to SDS–PAGE. In addition to 2E2, at least five other P450 forms have been identified in the liver of neonatal rabbits, including 1A1, 1A2, 2B4, 2C3, and 3A6 (15). The purification of 2E2 from neonatal liver microsomes to electrophoretic homogeneity (Fig. 1) is summarized in Table I. The liver of 2-week-old rabbits has a much lower content of total microsomal protein than that of adult liver (8 vs 50 mg/g of tissue) and a lower specific content of total microsomal P450, but has a relatively high content of lipids. To achieve optimal solubilization, it was necessary to dilute the microsomal preparation to a final concentration of less than 1.5 mg of protein/ml with NP-10 and cholate present at 1% each. Perhaps as a result of the high fat content and the stringent solubilization conditions, most of the solubilized microsomal P450 failed to precipitate with 14% PEG. The fraction precipitating with 7 to 14% PEG had about 30% of the total starting microsomal P450, but was not suitable for further processing because it could not be resolubilized. In the typical experiment shown, the overall yield based on starting total P450 was 2.7%. The final preparation of

FIG. 1. SDS–PAGE of microsomes and purified P450 preparations. Protein samples were applied to a 7.5% polyacrylamide gel. Migration was from top to bottom, and visualization was by silver staining. Lane a, hepatic microsomal proteins from 2-week-old rabbits, 1.7 μg of protein; lanes b and d, purified 2E2 from neonatal rabbits and purified 2E1 from adult rabbits, respectively, 0.1 μg of protein; and lane c, a 1:1 mixture of purified 2E1 and purified 2E2, 0.1 μg of total protein.

FIG. 2. Immunoblot analysis of microsomes and purified P450 preparations. Protein samples were submitted to electrophoresis in 7.5% acrylamide gels and electrophoretically transferred to nitrocellulose sheets. For immunochemical detection of 2E protein, the nitrocellulose sheets were treated with polyclonal (experiment 1) or monoclonal antibodies to rabbit 2E1 (experiment 2), or with a monoclonal antibody (1-98-1) to rat 2E1 (experiment 3). Lane a, hepatic microsomal proteins from 2-week-old rabbits, 5 μg of protein; and lanes b and c, purified 2E2 and 2E1, respectively, 0.1 μg of protein.

2E2 had a content of 15.9 nmol of heme per milligram of protein and comigrated with purified 2E1 on SDS–PAGE (Fig. 1).

In other experiments, a mixture of 2E1 and 2E2 was purified from liver microsomes of acetone-treated adult rabbits in the presence of 4-MP. When the same purification procedure was carried out in the absence of 4-MP, however, only 2E1 was obtained in the final preparation. Thus, stabilization with 4-MP leads to the retention of 2E2 in the final preparation from adult animals, but attempts to separate the two proteins have so far been unsuccessful.

NH₂-terminal sequence analysis. Cytochrome 2E2 is distinguished from 2E1 by the amino acid at position 14 of the NH₂-terminus, which is Ala in 2E2 and Val in 2E1 (3). The first 26 amino acid residues in the NH₂-terminus of the purified neonatal 2E2 were determined and found to agree with those deduced from the genomic sequence of 2E2 (3), with Ala at position 14 and only a trace of Val that was barely above background. The predicted NH₂-terminal Met was not present in the 2E2 protein, nor was it found in the 2E1 protein (4). The first three residues in the NH₂-terminus (Ala–Val–Leu) were absent in approximately 30% of the purified 2E2 preparation, whereas 50% of the P450 2y preparation did not possess these three amino acid residues (15). The lower amount of degraded protein and the absence of P420 in the highly purified preparation of 2E2 reported in the present study suggest that 4-MP not only prevents the conversion of 2E2 to P420, but also protects the enzyme to some extent from proteolytic degradation. The importance of these three residues to the activity as well as stability of the cytochrome remains to be determined, but a recent study from this laboratory has shown that removal of the NH₂-terminal 29 residues of 2E1 did not affect its activity toward ethanol or N,N-nitrosodiethylamine (38).
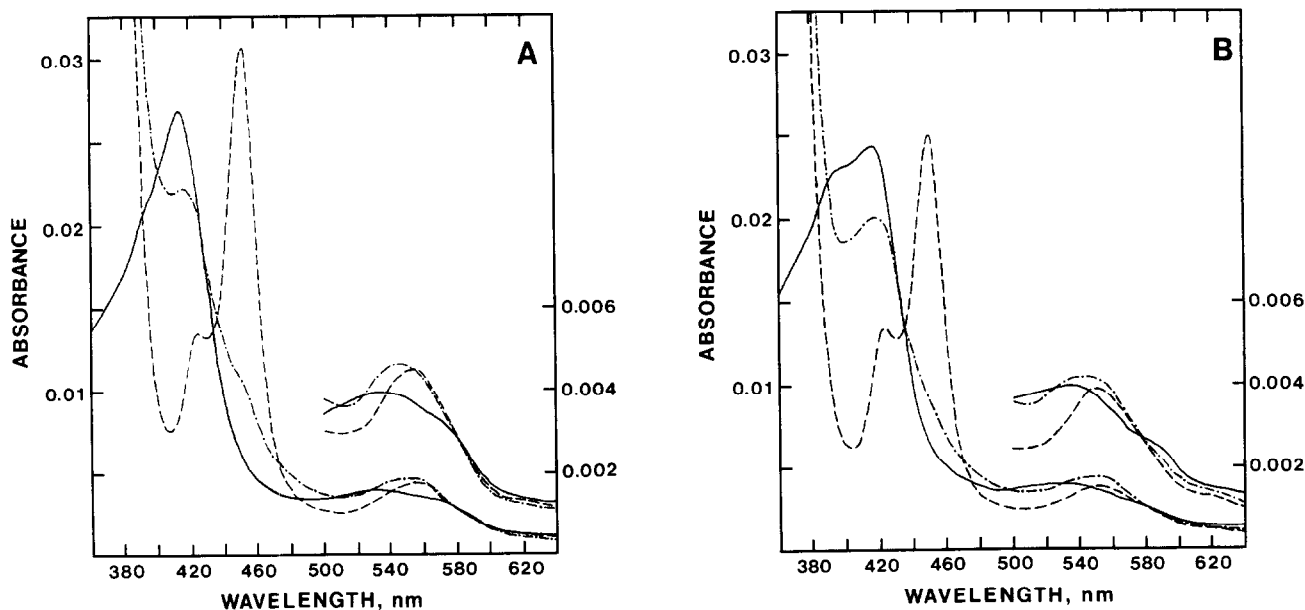
FIG. 3. Absolute spectra of purified 2E2 from neonatal rabbits. The concentration of 2E2, based on heme analysis, was 0.25 nmol per milliliter of 0.1 M potassium phosphate buffer, pH 7.4, containing 20% (A) or 0.13% glycerol (B). (—) Oxidized, (- · · -) dithionite-reduced, (- - -) reduced-CO complex.
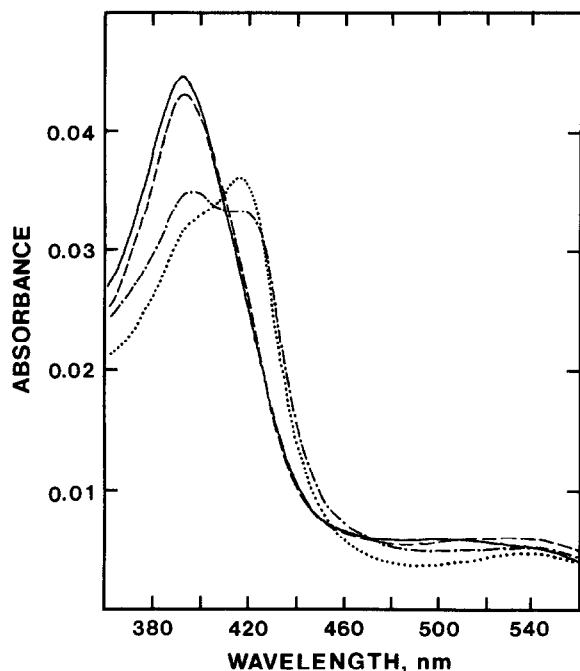
FIG. 4.   Absolute spectra of purified 2E1 and 2E2 from acetone-treated adult rabbits. The concentrations of the cytochromes, based on heme analysis, were 0.45 nmol per milliliter of 0.1 M potassium phosphate buffer, pH 7.4, containing varying amounts of glycerol. (—) 2E1 with 0.3% glycerol; (---) 2E1 with 20% glycerol; (-·-) copurified 2E1 and 2E2 in 1.6% glycerol; (·····) copurified 2E1 and 2E2 in 20% glycerol.

The first 30 NH$_2$-terminal residues of the mixed 2E1 and 2E2 preparation from acetone-treated adult rabbits were also determined and found to agree with those of 2E2 except at cycle 14, where a mixture of Ala and Val was found at a molar ratio of approximately 1:6. Cleavage of the three residues at the NH$_2$-terminus was also observed, but the fraction of the degraded protein was only about 6%. It is not clear if the apparently lower extent of degradation results from in vivo protection of the 2E proteins by acetone, or from an inherent resistance of 2E1 to proteolysis. In this connection, it is known that heterologously expressed 2E1 has greater stability than 2E2 during detergent solubilization and fractionation (13).

Immunochemical analysis.   Immunoblot analysis (Fig. 2) of neonatal microsomes and purified 2E2 was performed with three different antibodies to 2E1: a polyclonal, inhibitory antibody to rabbit 2E1; a monoclonal, noninhibitory antibody (1H.11) to rabbit 2E1; and a monoclonal antibody (1-98-1) to rat 2E1. All three antibodies recognized purified 2E2 as well as a single band with the same mobility in hepatic microsomes, thus establishing that purified 2E2 is immunochemically related to 2E1. In other experiments, the level of 2E2 in liver microsomes of 2-week-old rabbits was found to be approximately 25 to 30% of the total microsomal P450 based on immunoquantitation with purified 2E2 as a standard and the assumption that the level of 2E1 is negligible at this age.

In contrast, the combined level of 2E1 and 2E2 in adult rabbit liver is about 5% of the total microsomal P450 (39). Bonfils et al. (15) reported that the combined level of 1A1, 1A2, 2B4, 2C3, and 3A6 accounts for about 40% of total P450 at 2 weeks of age. Thus, 2E2 appears to be the principal hepatic P450 at this age.

Spectral properties.   As is shown in Fig. 3, glycerol, which is present in the storage buffer to stabilize P450, significantly alters the spectral properties of 2E2. Ferric 2E2 is predominantly low-spin in the presence of 20% glycerol, with an absorption coefficient of 108 mM^{-1} cm^{-1} for the absorbance maximum at 414 nm, but the cytochrome is in a mixed high- and low-spin state with absorbance maxima at 393 and 417 nm when the glycerol concentration is 0.13%. It is not known if 2E2 is completely high-spin in the absence of glycerol since removal of glycerol by dialysis results in formation of P420, which has maximal absorbance at 412 to 413 nm in the ferric state (data not shown). The absolute spectrum of dithionite-reduced 2E2 exhibits an absorption maximum at 415 nm with an absorption coefficient of 89 mM^{-1} cm^{-1} in the presence of 20% glycerol but displays an absorbance maximum at 420 nm with an absorption coefficient of 81 mM^{-1} cm^{-1} in the presence of 0.13% glycerol. A small

TABLE II

Monooxygenase Activities of 2E1 and 2E2[a]

Substrate	Activities	
	2E1	2E2
	(nmol/min/ nmol P450)	
N-Nitrosodimethylamine (10 mM)	8.1	3.0
N-Nitrosodiethylamine (10 mM)	14.8	8.9
N-Nitroso-N-methyl-N-phenylamine[b] (4 mM)	5.6	1.9
Anisole[b] (4 mM)	2.5	0.8
Ethylene glycol dimethyl ether (10 mM)	3.3	0.6
Hexamethylphosphoramide (10 mM)	6.8	4.0
Ethanol[c] (100 mM)	20.0	14.2
Aniline (3 mM)	29.2	8.9

[a] Unless otherwise indicated, the reaction mixtures contained 100 mM potassium phosphate buffer, pH 6.8, a reconstituted system with 0.05 μM P450, 0.15 μM NADPH–cytochrome P450 reductase, and 15 μg/ml phospholipid, and 1 mM NADPH. All reactions were carried out at 37°C. The concentrations of glycerol present were 15 mM with N-nitrosodimethylamine, N-nitroso-N-methyl-N-phenylamine, anisole, ethylene glycol dimethyl ether, or hexamethylphosphoramide, 35 mM with N-nitrosodiethylamine or aniline, and 70 mM with ethanol. The reaction product measured was p-aminophenol with aniline, acetaldehyde with N-nitrosodiethylamine or ethanol, or formaldehyde with the other substrates.

[b] Substrate was added in 4 μl of ethanol to a 1-ml final volume.

[c] The incubation mixture contained 50 mM phosphate buffer, pH 7.4.

TABLE III

Effect of b_5 on the Oxygenase Activities of 2E1 and 2E2[a]

		Activities	
Substrate	Molar ratio, b_5/P450	2E1	2E2
		(nmol/min/ nmol P450)	
N-Nitrosodimethylamine	0	5.0	2.8
(2.1 mM)	4	6.6	7.0
N-Nitrosodiethylamine	0	9.3	2.9
(2.0 mM)	8	8.7	6.2
	0	26.5	10.9
Butanol (15 mM)	8	10.8	8.7
	0	8.3	5.0
p-Nitrophenol (0.1 mM)	4	30.8	21.9

[a] Reaction mixtures contained 50 mM phosphate buffer, pH 7.4, except for p-nitrophenol hydroxylation, which was at pH 6.8. The concentrations of glycerol present are 15 mM with N-nitrosodiethylamine or p-nitrophenol, 35 mM with N-nitrosodimethylamine, and 70 mM with butanol. Other conditions were as in Table II.

shoulder was found at about 448 nm in the presence of 20% glycerol, but not at the lower glycerol concentration, and was more prominent at glycerol concentrations higher than 20% (not shown). The glycerol-induced absorbance change at 448 nm was also found with 2E1, although to a smaller extent. In other experiments with either 2E1 or 2E2, pronounced shoulders were observed at 446 to 448 nm in the presence of other ligands such as 4-MP or dimethyl sulfoxide. The absolute spectrum of reduced, CO-complexed 2E2 shows a small amount of P420 and an absorption maximum at 452 nm with an absorption coefficient of 124 mM^{-1} cm^{-1} in the presence of 20% glycerol and 101 mM^{-1} cm^{-1} in the presence of 0.13% glycerol. The reduced carbonyl difference spectrum of 2E2 (not shown) exhibits a maximum at 452 nm with an absorption coefficient of 84 mM^{-1} cm^{-1} in the presence of 20% glycerol and 71 mM^{-1} cm^{-1} in the presence of 0.13% glycerol. Thus, glycerol affects the spin state and the absorption coefficients of ferric, ferrous, and CO-complexed ferrous 2E2 in a concentration-dependent manner. In the report by Bonfils et al. (15), the partially purified P450 2y (with a specific content of 8 nmol/mg of protein) had an absorbance maximum at 418 nm in the oxidized state, 422 nm in the reduced state, and 452 nm in the CO-complexed state. However, it was not indicated whether glycerol was present.

The absolute spectra of purified 2E1 and of the mixture of 2E1 and 2E2 from acetone-treated adult rabbits are shown in Fig. 4. Notably, the oxidized spectrum of 2E1 does not show a significant spin shift (high- to low-spin) when recorded in the presence of 0.3% or 20% glycerol, although a small decrease in the absorbance at 393 nm is observed with 20% glycerol. This is in contrast to the spectrum of the mixed 2E1/2E2 preparation, which shows

a high- to low-spin shift when the glycerol concentration is increased from 1.6 to 20%.

Catalytic properties. The substrate specificity of purified 2E2 was studied in the reconstituted enzyme system containing NADPH, NADPH–cytochrome P450 reductase, and phophatidylcholine. As shown in Table II, 2E2 is active toward all of the substrates tested, although less so than is 2E1 under identical conditions. The ratio of activities for 2E1 to 2E2 varies from 1.4 for ethanol to 5.5 for ethylene glycol dimethyl ether. Glycerol, which is known to be a weak inhibitor (40, 41) of rat 2E1, was present in the incubation mixtures as a stabilizing agent for the enzymes, as indicated in the legend to the table. Therefore, the observed activity differences may be due, at least in part, to differential inhibition by glycerol. The results presented clearly show that 2E2 catalyzes the reactions already identified for 2E1, but further studies will be required to establish the detailed kinetic differences between the two isozymes.

The effect of b_5 on the activities of 2E2 was examined with four different substrates, with the results shown in Table III. Differential effects of b_5 on the activities of 2E1 and 2E2 were found. For instance, b_5 did not have a significant effect on the 2E1-catalyzed dealkylation of N-nitrosodiethylamine or N-nitrosodimethylamine at the substrate concentrations used, but increased the activity of 2E2 more than twofold with either substrate. Conversely, b_5 inhibited 2E1 activity with butanol by 60% but 2E2 activity by only 20%. Nevertheless, the extent of stimulation of the p-nitrophenol hydroxylation activity of 2E1 and 2E2 by b_5 was similar. Interestingly, the ratio of activities of 2E1 to 2E2 was reduced to nearly unity (0.9–1.4) in the presence of b_5 for all four substrates. In experiments not presented, both 2E1- and 2E2-catalyzed butanol oxidation were inhibited by a polyclonal anti-2E1 antibody by about 90% at a ratio of 10 mg of IgG/ nmol of P450. Cytochrome 2E1 was consistently more active than 2E2 with butanol as a substrate, whether the reaction was conducted at pH 6.4 or 7.7, or at substrate

TABLE IV

Stoichiometry of 2E-Catalyzed NADPH Oxidase Reaction[a]

Enzyme	NADPH oxidized (a)	O_2 consumed (b)	H_2O_2 formed (c)	Molar ratio, NADPH/ O_2/H_2O_2	(a) − (c) (b) − (c)
	(nmol/ml reaction mixture)				
2E1	141 ± 0	89 ± 8	25 ± 1	1.58:1.0:0.28	1.8
2E2	138 ± 4	89 ± 4	16 ± 1	1.55:1.0:0.18	1.7

[a] Duplicate determinations were made for each isozyme. The experimental values are given as the mean and standard deviation. In other experiments, the ratio of O_2 consumed to H_2O_2 formed during turnover of glucose oxidase with β − D (+)-glucose was 0.95. The concentration of glycerol in the reaction mixtures containing 2E1 and 2E2 was 67 and 207 mM, respectively.

concentrations ranging from 0.6 to 15 mM without b_5 but in the presence of 70 mM glycerol.

NADPH oxidase activity in the absence of substrate. Previous investigation of the stoichiometry of the P450-catalyzed NADPH oxidase reaction in the absence of substrate indicated that 2E1 utilizes a small fraction of NADPH-derived reducing equivalents for H_2O_2 formation, with most of the NADPH presumably being oxidized in the four-electron reduction of molecular oxygen to water (9). In the present study, stoichiometric determinations were conducted with both 2E1 and 2E2 in a reconstituted system under endpoint conditions as previously described (9). After addition of a limiting amount of NADPH, O_2 consumption was followed to completion of the reaction, which took 13 and 26 min with 2E1 and 2E2, respectively. While the rate of oxygen consumption for 2E2 is lower than that seen for 2E1, 2E2 is nevertheless more active than the other P450 isozymes previously examined. The extent of O_2 consumption was the same for both isozymes as illustrated in Table IV, but the amount of H_2O_2 formed with 2E2 was about two-thirds of that with 2E1. The two-electron oxidase activity of 2E1 or 2E2 accounted for less than one-fifth of the NADPH oxidized. When the amounts of NADPH oxidized and O_2 consumed were taken into account for H_2O_2 formation, it was apparent that, in addition, nearly 2 mol of NADPH were oxidized for each mole of O_2 consumed. Taken together, the data indicate that the rapid consumption of O_2 by the 2E isozymes in the absence of a hydroxylatable substrate is mostly associated with H_2O formation.

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REFERENCES

1. Koop, D. R., and Coon, M. J. (1986) *Alcohol. Clin. Exp. Res.* **10**, 44S–49S.

2. Nebert, D. W., Nelson, D. R., Coon, M. J., Estabrook, R. W., Feyereisen, R., Fujii-Kuriyama, Y., Gonzalez, F. J., Guengerich, F. P., Gunsalus, I. C., Johnson, E. F., Loper, J. C., Sato, R., Waterman, M. R., and Waxman, D. J. (1991) *DNA Cell Biol.* **10**, 1–14.

3. Khani, S. C., Porter, T. D., Fujita, V. S., and Coon, M. J. (1988) *J. Biol. Chem.* **263**, 7170–7175.

4. Koop, D. R., Morgan, E. T., Tarr, G. E., and Coon, M. J. (1982) *J. Biol. Chem.* **257**, 8472–8480.

5. Morgan, E. T., Koop, D. R., and Coon, M. J. (1982) *J. Biol. Chem.* **257**, 13,951–13,957.

6. Yang, C. S., Yoo, J. S. H., Ishizaki, H., and Hong, J. (1990) *Drug Metab. Rev.* **22**, 147–159.

7. Vaz, A. D. N., Roberts, E. S., and Coon, M. J. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 5499–5503.

8. Koop, D. R., and Casazza, J. P. (1985) *J. Biol. Chem.* **260**, 13,607–13,612.

9. Gorsky, L. D., Koop, D. R., and Coon, M. J. (1984) *J. Biol. Chem.* **259**, 6812–6817.

10. Ekstrom, G., and Ingelman-Sundberg, M. (1989) *Biochem. Pharmacol.* **38**, 1313–1319.

11. Tindberg, N., and Ingelman-Sundberg, M. (1989) *Biochemistry* **28**, 4499–4504.

12. Porter, T. D., Khani, S. C., and Coon, M. J. (1989) *Mol. Pharmacol.* **36**, 61–65.

13. Pernecky, S. J., Porter, T. D., and Coon, M. J. (1990) *Biochem. Biophys. Res. Commun.* **172**, 1331–1337.

14. Koop, D. R. (1990) *Chem. Res. Toxicol.* **3**, 377–383.

15. Bonfils, C., Combalbert, J., Pineau, T., Angevin, J., Larroque, C., Derancourt, J., Capony, J.-P., and Maurel, P. (1990) *Eur. J. Biochem.* **188**, 187–194.

16. Peng, H.-M., Porter, T. D., Ding, X., and Coon, M. J. (1991) *Mol. Pharmacol.* **40**, 58–62.

17. Coon, M. J., van der Hoeven, T. A., Dahl, S. B., and Haugen, D. A. (1978) *in* Methods in Enzymology (Fleischer, S., and Packer, L., Eds.), Vol. 52, pp. 109–117, Academic Press, San Diego.

18. Koop, D. R., and Coon, M. J. (1984) *Mol. Pharmacol.* **25**, 494–501.

19. Thurman, R. G., Ley, H. G., and Scholz, R. (1972) *Eur. J. Biochem.* **25**, 420–430.

20. Robinson, J., and Cooper, J. M. (1970) *Anal. Biochem.* **33**, 390–399.

21. Bernt, E., and Bergemeyer, H. U. (1974) *in* Methods of Enzymatic Analysis (Bergemeyer, H. U., Ed.), Vol. 4, pp. 2246–2248, Academic Press, New York.

22. Gorsky, L. D., and Coon, M. J. (1986) *Drug Metab. Dispos.* **14**, 89–96.

23. Koop, D. R. (1986) *Mol. Pharmacol.* **29**, 399–404.

24. Nash, T. (1953) *Biochem. J.* **55**, 416–421.

25. Koop, D. R., Nordblom, G. D., and Coon, M. J. (1984) *Arch. Biochem. Biophys.* **235**, 228–238.

26. Ding, X., and Coon, M. J. (1988) *Drug Metab. Dispos.* **16**, 265–269.

27. Mieyal, J. J., Ackerman, R. S., Blumer, J. L., and Freeman, L. S. (1976) *J. Biol. Chem.* **254**, 3436–3441.

28. Omura, T., and Sato, R. (1964) *J. Biol. Chem.* **239**, 2370–2378.

29. Antonini, E., and Brunori, M. (1971) *in* Hemoglobins and Myoglobins in Their Reactions with Ligands (Neuberger, A., and Tatum, E. L., Eds.) North-Holland, London.

30. Hewick, R. M., Hunkapillar, M. W., Hood, L. E., and Dreyer, W. J. (1981) *J. Biol. Chem.* **256**, 7990–7997.

31. Ding, X., Koop, D. R., Crump, B. L., and Coon, M. J. (1986) *Mol. Pharmacol.* **30**, 370–378.

32. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.

33. Haugen, D. A., and Coon, M. J. (1976) *J. Biol. Chem.* **251**, 7929–7939.

34. Strittmatter, P., Fleming, P., Connors, M., and Corcoran, D. (1978) *in* Methods in Enzymology (Fleischer, S., and Packer, L., Eds.), Vol. 52, pp. 97–101, Academic Press, San Diego.

35. French, J. S., and Coon, M. J. (1979) *Arch. Biochem. Biophys.* **195**, 565–577.

36. Park, S. S., Ko, I.-Y., Patten, C., Yang, C. S., and Gelboin, H. V. (1986) *Biochem. Pharmacol.* **35**, 2855–2858.

37. Ding, X., and Coon, M. J. (1988) *Biochemistry* **27**, 8330–8337.

38. Larson, J. R., Porter, T. D., and Coon, M. J. (1991) *J. Biol. Chem.* **266**, 7321–7324.

39. Koop, D. R., Crump, B. L., Nordblom, G. D., and Coon, M. J. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 4065–4069.

40. Clejan, L. A., and Cederbaum, A. I. (1990) *Biochem. Biophys. Acta* **1034**, 233–237.

41. Yoo, J. S. H., Cheung, R. J., Patten, C., Wade, D., and Yang, C. S. (1987) *Cancer Res.* **47**, 3378–3383.