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PURIFICATION AND CHARACTERIZATION OF HUMAN LIVER CYTOCHROME P-450-ALC*

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SUMMARY: Cytochrome P-450-ALC, an ethanol-oxidizing form of microsomal cytochrome P-450 (P-450), has been purified from human liver. P-450-ALC (Mr = 54,000 daltons) is a low-spin ferric hemeprotein with a CO-reduced Soret maximum at 452 nm, and has an NH_2-terminal amino acid sequence nearly identical to that deduced from a human P-450-ALC cDNA clone. In a reconstituted system, P-450-ALC oxidizes ethanol and aniline at turnover rates (12.2 and 7.3 nmol min^{-1}, respectively) 10-fold greater than two other human P-450 isozymes (termed P-450-B and P-450-C) purified from the same liver. Both P-450-ALC and P-450-C effectively demethylate N-nitrosodimethylamine (NDMA) at low substrate concentrations (0.5 mM), especially in the presence of cytochrome b_5. Our results provide direct evidence for a liver P-450 isozyme in humans with catalytic properties similar to the related alcohol-inducible rodent P-450s and also reveal a new human NDMA demethylase.

The functional properties of cytochrome P-450-ALC[2], a unique ethanol (ETOH)-inducible isozyme of liver microsomal P-450, have generated much interest. Among these properties are the enhanced capacity of rodent P-450-ALC to oxidize ETOH and other primary alcohols (1-3) and to bioactivate NDMA, a ubiquitous carcinogen (4-6). Both purification and immunological studies have shown that P-450-ALC catalyzes a significant proportion of the alcohol oxidation occuring in liver microsomes from rodents treated with ETOH (3,7). These studies have also implicated rodent P-450-ALC as the primary, if

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[2]To avoid confusion, we have designated the liver cytochrome P-450 isozyme inducible by ethanol as P-450-ALC (23). This enzyme has been referred to as HLj and P-450j (in humans), form 3a (in rabbits), and P-450j, P-450et, and $P\text{-}450_{ac}$ (in rats).

Abbreviations used: ETOH, ethanol; P-450, liver microsomal cytochrome P-450; NDMA, N-nitrosodimethylamine; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; Fp, liver microsomal NADPH: cytochrome P-450 reductase; DLPC, synthetic dilauroylphosphatidylcholine.

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not only, P-450 isozyme that demethylates NDMA at physiologically-relevant substrate concentrations (5,6,8). Besides ETOH, such structurally-diverse agents as acetone, isoniazid, pyrazole, dimethylsulfoxide, and imidazole are potent inducers of P-450-ALC in rodents (8,9). The capacity of P-450-ALC to metabolize one of these agents, namely acetone, has led to the proposal that this enzyme plays an important role in ketone disposition (10).

Using antibodies prepared against a rodent form of P-450-ALC, we as well as others have recently described the existence of a immunochemically-related P-450 isozyme in man (11,12). The NH_2-terminal amino acid sequence of this human P-450, derived from a human P-450-ALC cDNA clone (13) and from an immunoaffinity-purified protein (12), exhibits significant homology with the NH_2-terminal sequences of the related rodent enzymes. Besides these structural and immunochemical similarities, however, it is not known at present whether human P-450-ALC also possesses those specific catalytic properties common to the rodent ethanol-inducible P-450s. In this report, we describe several catalytic features of purified human liver P-450-ALC.

EXPERIMENTAL PROCEDURES

Human liver (300 g) was from a renal trasplant donor (43 yr old male Causcasian) who died traumatically; the tissue was procured through the National Diabetes Research Interchange. The patient had no prior history of ETOH or drug abuse, and was not diabetic. Within 30 min of death, the liver was removed and quick-frozen in liquid nitrogen, followed by storage at -135°C.

Purification of Cytochromes P-450.

An outline of the purification procedures used is shown in Fig. 1. Briefly, microsomes were prepared (14) from the human liver specimen and were solubilized with CHAPS (2 mg/mg protein). Chromatography of solubilized microsomes on Tryptamine-Sepharose was performed essentially as described by Lu et al. (15) but incorporated buffer modifications proving more effective for resolution of human P-450s. Elution of the column with a buffer containing 0.5% cholate plus 1.0% Lubrol PX yielded, upon SDS-PAGE analysis (16), a protein fraction highly enriched in P-450-ALC and two additional P-450 isozymes (arbitrarily designated B and C).

After simultaneous concentration and removal of two major non-P-450 contaminants using hydroxylapatite (Hypatite C), the partially-purified P-450-ALC fraction was subjected to DE-53 chromatography. This anion-exchange step resolved P-450-B from both P-450-ALC and P-450-C but did not completely separate the latter two proteins. P-450-C was then removed from the P-450-ALC preparation by immunoaffinity chromatography on an agarose resin to which monospecific polyclonal anti P-450-C IgG (preparation described below) had been covalently attached. P-450-C, enriched in the second A_{417} peak from the DE-53 column (Fig. 1), was resolved from P-450-ALC using CM-Sepharose chromatography. CM-Sepharose was also used to eliminate residual contaminants present in the P-450-B sample. Finally, excess detergent was removed from all three enzyme preparations by chromatography on hydroxylapatite.

Prior to P-450 elution, washing of the charged Tryptamine-Sepharose column with a buffer containing 0.5% cholate and 0.5 M KCl yielded a protein fraction enriched in both Fp and cytochrome b_5. Fp was further purified

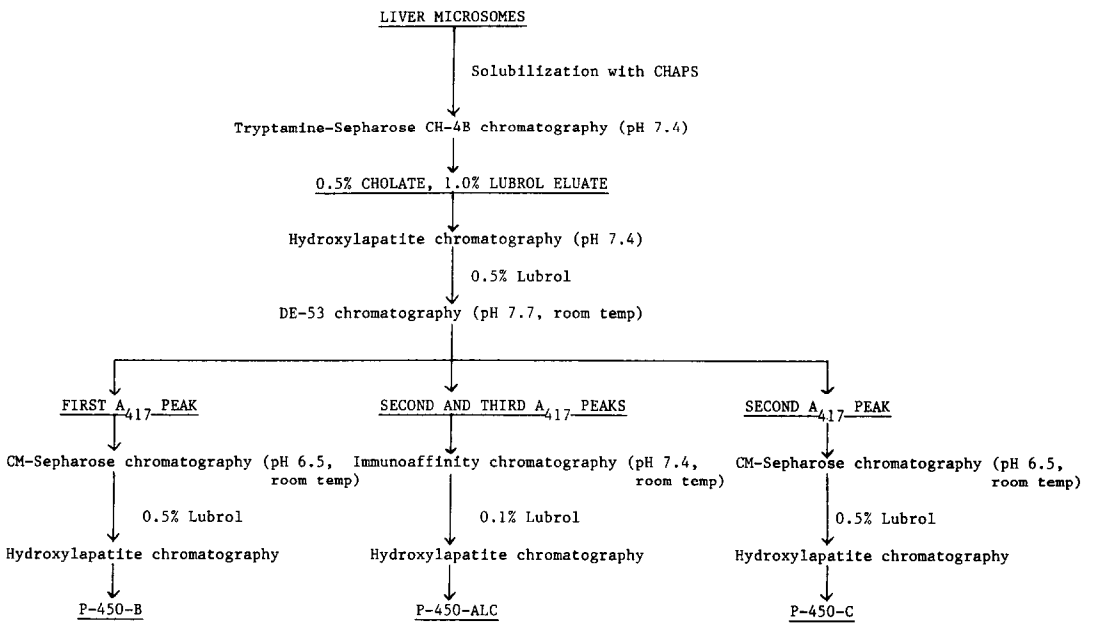

Fig. 1. Scheme for the purification of cytochromes P-450-ALC, P-450-B and P-450-C from human liver microsomes. The procedures were performed at 4°C unless denoted otherwise. All chromatography and dialysis buffers contained 1 mM DTT, 1 mM EDTA and 20% glycerol.

using 2',5'-ADP agarose according to Ardies et al. (17); the electrophoretically-homogenous Fp preparation had a final specific activity of 20,700 units (nmol cytochrome c reduced/min/mg protein at 22°C). Cytochrome b_5 was purified to a specific content of 29.7 nmol/mg protein employing a combination of hydroxylapatite and DE-52 chromatography.

Enzyme Assays.

ETOH oxidase activity was determined as previously described (1,18). Demethylation of [^{14}C] NDMA was assessed according to Levin et al. (5). Aniline hydroxylation and aminopyrine N-demethylation were measured as described by Ryan et al. (3) and Guengerich et al. (19), respectively. Reaction mixtures contained human liver microsomes (0.2 nmol P-450; 0.5 mg protein) or a reconstituted system (0.1 nmol P-450, 900 units Fp and 30 µg DLPC), 100 mM potassium phosphate buffer, 1 mM NADPH and substrate (50 mM ETOH, 0.5 mM NDMA, 2.5 mM aniline, or 5 mM aminopyrine) in a final volume of 1.0 ml (0.5 ml for NDMA demethylase assays and a proportional amount of enzyme). Incubation mixture pH was 7.4 (ETOH and aminopyrine), 7.0 (aniline) or 6.8 (NDMA). Reactions were initiated with NADPH and terminated after 10 min at 37°C.

Other Methods.

Antisera to purified P-450-C was raised in rabbits (3) and the IgG fraction purified using Protein A-Sepharose. NH_2-terminal sequencing was performed using an Applied Biosystems 470A gas-phase sequenator with an on-line PTH amino acid analyzer (Applied Biosystems 120). The purified proteins (30 µg) were precipitated with methanol, freed of non-protein solutes, redissolved in neat trifloroacetic acid, and then applied to the sequenator.

RESULTS

As shown in Fig. 2, P-450-ALC as well as P-450-B and P-450-C were purified to near homogeneity from a single preparation of human liver microsomes. The electrophoretic mobility of P-450-ALC is clearly distinct from the other two P-450s (Fig. 2, lane 6). Subunit molecular weights of these proteins are presented in Table I, as are their spectral properties. Each P-450 is a low-spin ferric hemeprotein with different Soret maxima in the oxidized as well as ferrous carbonyl states.

The catalytic properties of P-450-ALC, P-450-B and P-450-C are described in Table II. Of the three hemeproteins, only P-450-ALC was an effective catalyst of ETOH oxidation and aniline hydroxylation. In contrast, P-450-ALC did not N-demethylate aminopyrine whereas P-450-B and, to a lesser extent, P-450-C promoted this reaction. Both P-450-ALC and P-450-C metabolized NDMA at low substrate concentrations; inclusion of human cytochrome b_5 into the reconstituted system markedly stimulated the NDMA demethylase activity of each enzyme.

The NH_2-terminal amino acid sequence of P-450-ALC exhibits limited homology (<30%) with either P-450-B or P-450-C over the initial 16 residues

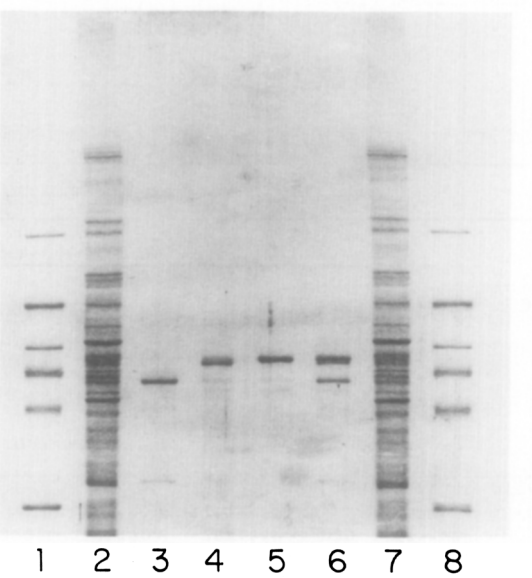

Fig. 2. SDS-PAGE of human liver microsomes and purified cytochromes P-450. Samples were analyzed on a slab gel 0.75 mm thick containing 7.5% acrylamide using the discontinuous buffer system (16). Migration proceeds from top to bottom. Lanes 2 and 7, microsomes (10 µg); lanes 3,4 and 5, cytochrome P-450-B, P-450-ALC and P-450-C, respectively, (0.5 ug); lane 6, mix of all three P-450s (0.25 µg each); lanes 1 and 8, protein standards with molecular weights of 98,000, 68,000, 58,000, 53,000, 43,000 and 29,000 (0.5 µg each).

TABLE I

CHARACTERIZATION OF HUMAN LIVER CYTOCHROMES P-450-ALC, P-450-B, and P-450-C

	P-450-ALC	P-450-B	P-450-C
Monomeric molecular weight	54,000	50,000	55,000
Specific content (nmol/mg protein)	7.2	6.8	14.6
CO-reduced difference spectrum; Soret maximum (nm)	452	452.5	451
Absolute oxidized spectrum; Soret, β-, and α-band maxima (nm)	418,538,566	417,535,568	416,534,568

The hemeproteins were purified as described under Experimental Procedures. Molecular weights were calculated from the calibrated SDS-polyacrylamide gel shown in Fig. 2. The concentration of cytochrome P-450 used to determine the absolute spectra ranged from 2 to 6 μM. The CO-reduced difference (20) and absolute spectra were generated in 100 mM potassium phosphate buffer (pH 7.4), 0.1 mM EDTA and 20% glycerol (plus 0.25% cholate and 0.2% Lubrol for the ferrous carbonyl difference spectrum of P-450-ALC). All spectra were recorded with an Aminco DW-2a spectrophotometer.

(Fig. 3). Except for the NH_2-terminal residue, the sequence of P-450-ALC is identical to both the reported sequence of HLj (12) and the deduced sequence of a human P-450j cDNA clone (13).

TABLE II

CATALYTIC ACTIVITIES OF PURIFIED HUMAN LIVER CYTOCHROMES P-450

	Substrate			
Hemeprotein	ETOH	Aniline	NDMA	Aminopyrine
	nmol product formed/min/nmol P-450[a]			
Liver Microsomes	12.6	1.6	3.2	16.9
P-450-ALC	12.2	7.3	2.8 (6.4)[b]	<1.0
P-450-B	1.2	<0.5	<0.04 (<0.04)	18.0
P-450-C	1.0	0.7	1.5 (7.0)	3.4

The catalytic activities of human P-450-ALC, P-450-B and P-450-C were determined using 0.1 nmol of each hemeprotein, saturating amounts of human liver Fp (900 units) and optimal concentrations of synthetic dilauroylphosphatidylcholine (50 μM). In reactions containing human liver microsomes (from which the three P-450 isozymes were isolated), an amount of protein equivalent to 0.2 nmol P-450 was used. Incubations were performed in 1.0 ml 100 mM potassium phosphate buffer for 10 min at 37°C; the pH of the reaction mixtures and the substrate concentration utilized are described under Experimental Procedures.

[a]Values represent the mean of at least 3 determinations.

[b]Values in parentheses represent NDMA demethylase activities determined in the presence of 0.4 μmol human liver cytochrome b_5.

CYTOCHROME	Residue 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
P-450-B	Met	Glu	Pro	Phe	Val	Val	Leu	Val	Leu	-	Leu	Ser	Ser	Met	Leu	Leu	Ser		
P-450-C	Met	-	Ser	Leu	Val	Val	Leu	Val	Leu	Leu	Leu	Ser	-	Leu	Leu	Leu			
P-450-ALC	Met	Ala	Leu	Gly	Val	Thr	Val	Ala	Leu	Leu	Val	Trp	Ala	Ala	Phe	Leu	Leu	Leu	Val
HLj	Ala	Ala	Leu	Gly	Val	Thr	Val	Ala	Leu	Leu	Val	Trp	Ala	Ala	Phe	Leu	Leu	Leu	
Human P-450j	(Met) Phe	Ala	Leu	Gly	Val	Thr	Val	Ala	Leu	Leu	Val	Trp	Ala	Ala	Phe	Leu	Leu	Leu	Val

Fig. 3. NH_2-terminal amino acid sequences of human P-450-ALC, P-450-B, P-450-C and related proteins. The NH_2-terminal sequences of cytochrome P-450-ALC, P-450-B and P-450-C were determined as described in Experimental Procedures. The NH_2-terminal sequences of HLj (12) and human P-450j (13) are given for comparison, and those residues identical to P-450-ALC are boxed. Whether the NH_2-terminal Met of human P-450j is present in the native protein is not known, since the sequence was deduced from a cDNA clone (13). Underlined P-450-B and P-450-C residues indicate those common to P-450-ALC.

DISCUSSION

In this report, we demonstrate for the first time that human P-450-ALC is related to the ETOH-inducible rodent P-450 isozymes not only in terms of structure but in catalytic properties as well. The NH_2-terminal amino acid sequence of the human hemeprotein we have purified is 57% and 49% homologous, respectively, with rat P-450j and rabbit LM-3a (3) over the first 19 amino acids. Human P-450-ALC, like it's rodent homologs (1-3), is a very effective catalyst of both ethanol oxidation and aniline hydroxylation. Neither of the two other human P-450 isozymes studied here, P-450-B and P-450-C, catalyzed these reactions to any appreciable extent. In addition,P-450-ALC was found to demethylate NDMA at a physiologically-relevant substrate concentration. Similar to rat P-450j (5,6), the NDMA demethylase activity of human P-450-ALC was stimulated several-fold in the presence of cytochrome b_5. P-450-C also exhibited NDMA demethylase activity, especially when reconstituted with cytochrome b_5. In contrast to rats (5,6), humans may therefore possess two structurally- and immunochemically-distinct liver P-450s that participate in NDMA bioactivation. Interestingly, the NH_2-terminal amino acid sequence of P-450-C is very similar to $P\text{-}450_{MP\text{-}1}$ and $P\text{-}450_{MP\text{-}2}$, two mephenytoin-metabolizing forms of human P-450 (21), although the reported molecular weights and spectral properties differ somewhat. Whether $P\text{-}450_{MP\text{-}1}$ and/or $P\text{-}450_{MP\text{-}2}$ catalyze NDMA demethylation has not been described.

Importantly, human P-450-ALC is an ETOH oxidase. This catalytic property, when considered together with the reported inducibility of the enzyme in humans by ETOH (11,12), suggest that P-450-ALC may play an important role in the metabolic tolerance to ETOH commonly observed in alcoholics (22). Studies designed to examine the role of P-450-ALC in the toxicity of alcohols,

commonly-used analgesics and environmental carcinogens in man are now in progress.

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