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Alcohol Oxidation by Isozyme 3a of Liver Microsomal Cytochrome P-450[1]

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COON, M. J., D. R. KOOP AND E. T. MORGAN. *Alcohol oxidation by isozyme 3a of liver microsomal cytochrome P-450.* PHARMACOL BIOCHEM BEHAV **18:** Suppl. 1, 177–180, 1983.—Liver microsomes from rabbits treated chronically with ethanol were solubilized and fractionated to yield a new isozyme of cytochrome P-450 in a homogeneous state. This cytochrome, designated as isozyme 3a on the basis of its relative electrophoretic mobility, is distinct from the known isozymes as judged by its spectral properties, minimal molecular weight, amino acid composition, and NH_2- and COOH-terminal amino acid sequences. In addition, peptide mapping by high performance liquid chromatography following trypsinolysis indicates that form 3a is a unique gene product. This cytochrome has unusually high activity in the oxidation of ethanol and other alcohols to aldehydes and in the *p*-hydroxylation of aniline as compared with the other isozymes of P-450. The ethanol-oxidizing activity of isozyme 3a, which requires the presence of NADPH and NADPH-cytochrome P-450 reductase and is stimulated by the presence of phosphatidylcholine, is not due to contamination by catalase or an NAD^+- or $NADP^+$-dependent alcohol dehydrogenase.

Alcohol metabolism Cytochrome P-450 Aniline *p*-hydroxylation Ethanol oxidation

CYTOCHROME P-450 is a remarkably versatile catalyst with an almost unlimited number of substrates. The chemical reactions attributed to liver microsomal cytochrome P-450 (P-450LM) include aliphatic and aromatic hydroxylation, N-and S-oxidation, epoxidation, oxidative removal of alkyl groups attached to nitrogen, sulfur, or oxygen atoms, peroxidation, deamination, dehalogenation, desulfuration, and reductive processes involving electron transfer to azo and nitro groups and to N-oxides and epoxides. The list of substrates includes physiologically occurring lipids such as fatty acids, prostaglandins, and steroids, as well as a huge number of foreign substances including pesticides, anesthetics, drugs, petroleum products, carcinogens, and organic solvents.

The present paper is concerned with the question of whether ethanol is also a substrate of P-450LM, or rather one or more specific isozymes of P-450LM. Some years ago we solubilized the P-450LM-containing enzyme system and resolved it into three components: cytochrome P-450, NADPH-cytochrome P-450 reductase, and phosphatidylcholine [16, 17, 27]. Evidence was subsequently obtained that substrate specificity in the reconstituted system resides in the different cytochrome fractions obtained after the administration of various inducing agents [18,24]. More definitively, the purification of several individual forms of the cytochrome enabled them to be characterized as proteins with different chemical and physical properties which could properly be called isozymes [7]. Of particular interest, the various isozymes, whether inducible or noninducible, have partly distinct, overlapping substrate specifities [3, 7, 19].

Whether P-450 plays a role in the oxidation of ethanol to acetaldehyde has been a controversial question [4, 8, 20, 29]. Some of the effects of chronic administration of ethanol to rats, including proliferation of the smooth endoplasmic reticulum of the liver and an increase in the P-450 content are well established, and an increased rate of microsomal aniline hydroxylation and alcohol oxidation have been reported [9, 10, 14, 21, 25, 26, 28, 30]. Lieber and DeCarli [14] concluded that a hepatic microsomal ethanol-oxidizing system occurs which is distinct from catalase and alcohol dehydrogenase, and the increased alcohol oxidation in microsomes from animals treated with ethanol was considered to be due to cytochrome P-450 [21, 23, 25, 30]. Reports appeared that the ethanol-oxidizing activity in liver microsomes could be solubilized and reconstituted [25] and that partial purification of the enzymes responsible could be achieved [23,30]. From these and other studies the intriguing possibility was raised that P-450 might bear a relationship to microsomal ethanol oxidation, but a firm conclusion could not be reached until the postulated isozyme was isolated and characterized. As recently reported elsewhere [12,22] and described in the present paper, we have identified and purified to homogeneity a unique form of P-450, designated isozyme 3a, from liver microsomes of rabbits chronically treated with ethanol. This isozyme has relatively high activity in the oxidation of ethanol and other alcohols as well as in the hydroxylation of aniline.

METHOD

P-450 isozymes 2, 3b, 3c, and 4 were isolated as described

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previously [5,13]. Isozyme 3a was purified from liver microsomes obtained from adult New Zealand male rabbits given drinking water containing 10% ethanol (v/v); the procedure, which is described in detail elsewhere [12], is an extension of that recently published for the purification of forms 3b and 3c from untreated rabbits [13]. The concentration of cytochrome P-450 in crude fractions was determined from the CO difference spectrum of the reduced protein with an extinction coefficient of 91 $mM^{-1}cm^{-1}$ for the difference between the maximum absorbance in the 450 nm region and at 490 nm. The concentration of the purified isozymes was determined from the absolute spectra of the ferrous carbonyl complexes with use of the following absorption coefficients: forms 2 and 3a at 451 and 452 nm, respectively, 110 $mM^{-1}cm^{-1}$; forms 3 b and 3c at 450 and 449 nm, respectively, 108 $mM^{-1}cm^{-1}$; and form 4 at 447 nm, 115 $mM^{-1}cm^{-1}$. The absolute spectrum of form 3a and the heme concentration were determined as previously described [13]. Electrophoretically homogeneous NADPH-cytochrome P-450 reductase was obtained from liver microsomes of phenobarbital-treated rabbits [6]. The preparations catalyzed the reduction of 45 to 55 μmol of cytochrome *c*/min/mg of reductase protein in the presence of 0.3 M potassium phosphate buffer, pH 7.7, at 30°C. Protein was determined by the method of Lowry *et al.* [15] as modified by Bensadoun and Weinstein [1]. Polyacrylamide slab gel electrophoresis was carried out in the presence of SDS with the discontinuous buffer system [12].

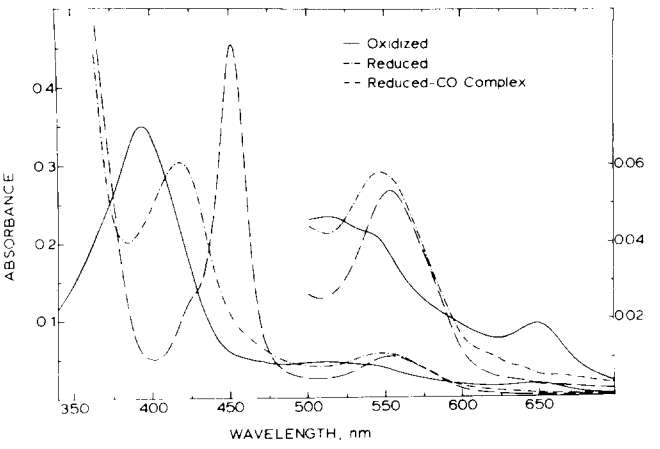

FIG. 1. Absolute spectra of purified isozyme 3a, taken from [12]. The concentration of the cytochrome, based on the heme content, was 4.2 μM in 150 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA. The spectra were recorded at 15°C. —, Oxidized; ·····, dithionite reduced; - - -, reduced-CO complex. Identical spectra were obtained in the presence of 0.5% Tergitol.

RESULTS

The spectral properties of isozyme 3a, which was homogeneous as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, are shown in Fig. 1. In the oxidized state the protein has a spectrum typical of high spin, pentacoordinate P-450 similar to form 4 but quite distinct from forms 2, 3b, 3c, and 6, which are isolated in the low spin state. The reduced and carbonyl forms have spectral characteristics typical of this cytochrome, with the CO complex having an absorption maximum at 452 nm.

Several lines of evidence indicate that isozyme 3a is distinct from the previously isolated forms of cytochrome P-450 from liver microsomes. These include the electrophoretic behavior as a measure of minimal molecular weight, the amino acid composition, and the NH_2- and COOH-terminal partial sequences [12]. Isozymes 2, 3b, and 3c all have an N-terminal methionine residue followed by aspartate or glutamate, followed in turn by a hydrophobic stretch of amino acids, and they possess as their C-terminal residues arginine, valine, and alanine, respectively. Isozyme 3a has a unique N-terminal sequence beginning with alanine and with a similar but different hydrophobic series of residues: Ala-Val-Leu-Gly-Ile-Thr-Val-Ala-Leu-Leu-Gly-Trp-Met-Val-Ile-Leu-Leu-Phe-Ile-Ser-Val-Trp-Lys-. The carboxyl terminal region is also unique, ending in leucine: -Arg-Ile-Val-Pro-Leu. As additional evidence that isozyme 3a is a unique gene product, peptide maps of the various cytochromes were compared by gel electrophoresis following treatment with several different proteases or by high performance liquid chromatography (HPLC) following exposure to trypsin [13]. The results obtained by the latter procedure are presented in Fig. 2. Forms 2, 3c, and 4 were very susceptible in the native form to trypsinolysis, but form 3a required the presence of a denaturant; 4 M urea was therefore included in the reaction mixtures. The results presented indicate that the peptide maps are unique for the various cytochromes whether determined at 214 nm or by fluorescence. We conclude, therefore, that the isozymes have significantly different primary structures.

We have previously observed that activities in ethanol oxidation and aniline *p*-hydroylation are considerably higher in microsomes from ethanol-treated rabbits than those from control or phenobarbital- or 5,6-benzoflavone-treated rabbits. The activities of the five cytochromes toward a series of substrates, taken from [22], are given in Table 1. Isozyme 3a had the highest activity toward ethanol, propanol, and butanol, and was 2.5 times as active as the next most active isozyme, LM_4, with ethanol as substrate, and 5.5 times as active as LM_2 towards butanol. In contrast, only low activities of all of the isozymes were observed with methanol as substrate. Another isozyme purified in this laboratory which we believe to be identical to form 6, first described by Johnson and Muller-Eberhard [11], had a turnover number of only 3.0 towards ethanol. The ethanol-oxidizing activity of the reductase in the absence of P-450 may contribute to the observed rates of ethanol oxidation in the presence of the various isozymes, but whether the presence of P-450 has an effect on this intrinsic activity of the reductase is not known. If we assume no such effect and correct the P-450 activities for the reductase present, the ethanol-oxidizing activity of LM_{3a} relative to the other isozymes becomes 3-fold higher than that of LM_2 and 4.5-fold higher than that of LM_4. LM_{3b} and LM_{3c} did not have significantly higher activities than the reductase alone. LM_{3a} had no detectable activity towards ethylmorphine, whereas the other four isozymes all had similar but low activities with this substrate. The constitutive isozymes, 3b and 3c [13], were the most active towards chlorcyclizine, and 3a was the least active. LM_2 was about 5-fold more active towards 7-ethoxycoumarin than LM_{3b}, the next most active isozyme, and LM_{3a} had only low but significant activity. The picture which emerges from these and other activity measurements is that the liver microsomal P-450's have somewhat different but overlapping substrate

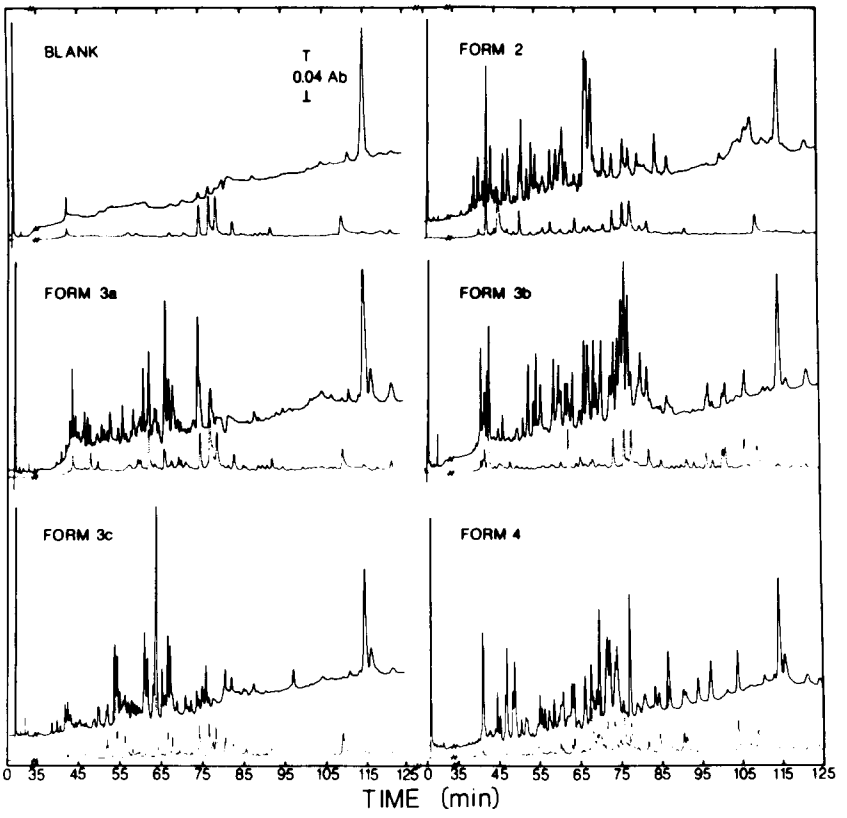

FIG. 2. Resolution of the tryptic peptides of forms 2, 3a, 3b, 3c, and 4 by HPLC. Each isozyme was treated with trypsin for 16 hr at 37°C in the presence of 4 M urea. The reactions were quenched by the addition of phosphoric acid, and 100 μl of each mixture (containing 100 μg of digested protein) were injected into the instrument. The chromatograms were developed using a program which regulated both the solvent composition and flow rate. The solid line represents the absorbance of the effluent at 214 nm, and the dotted line represents the fluorescence of the effluent with an excitation wavelength of 290 nm and a standard 370 filter. The fluorescence is in arbitrary units which are the same for each chromatogram. This figure is taken from [12].

specificities, and that 3a is the most selective of the various isozymes, having relatively high activity only toward alcohols and aniline.

DISCUSSION

The results described clearly indicate that a previously unrecognized form of P-450, isozyme 3a, has been isolated from rabbits chronically exposed to ethanol and that in the reconstituted enzyme system this isozyme has relatively high activity in the oxidation of ethanol to acetaldehyde. That our observed ethanol oxidizing activity of P-450LM$_{3a}$ is a P-450 catalyzed reaction, and not due to contaminating catalase or ADH, is supported by several lines of evidence. LM$_{3a}$ as isolated is an electrophoretically homogenous preparation [12]. The ethanol-oxidizing activity displays all of the characteristics of a typical P-450-catalyzed reaction, including a requirement for NADPH, reductase, and phospholipid. Catalatic activity of the preparation was not detected, and the reaction was not inhibited by azide. Moreover, catalase has little activity toward propanol or butanol [2]. Since oxidized pyridine nucleotides could not support the reaction, significant ADH activity can be excluded. Our findings,

TABLE 1

CATALYTIC ACTIVITY OF P-450LM$_{3a}$ COMPARED TO OTHER ISOZYMES*

Substrate	Catalytic activity of isozymes				
	2	3a	3b	3c	4
	nmol product formed/min/nmol P-450				
Methanol	1.3	1.7	1.1	1.1	1.9
Ethanol	3.6	10.8	1.2	1.6	4.4
l-Propanol	3.1	12.6	1.3	0.9	1.6
l-Butanol	2.5	14.1	0.7	0.4	1.9
Aniline	3.0	12.6	2.1	0.8	1.7
Ethylmorphine	4.6	<0.2†	4.9	3.0	2.6
Chlorcyclizine	1.5	0.4	6.4	5.0	1.0
7-Ethoxycoumarin	14.2	1.2	2.7	0	1.4

*Incubation conditions for each substrate were optimized using the most active isozyme for that substrate. Alcohol concentrations were: methanol, 116 mM; ethanol, 80 mM; propanol, 40 mM; and butanol, 22 mM.

†This value is at the limit of detection in the assay used for ethylmorphine.

therefore, give strong support for a microsomal pathway of ethanol oxidation, but the relative physiological significance of this process remains to be determined. Studies are in progress to determine by immunological and other techniques whether certain organelles, tissues, or species utilize the cytochrome P-450-dependent route for alcohol oxidation more extensively than others and also whether exposure to alcohol or other inducing agents increases the relative contribution of this pathway to alcohol metabolism.

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