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SHORT COMMUNICATIONS

Purification of Liver Microsomal Cytochrome P-448 from 3-Methylcholanthrene-Treated Rabbits

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SUMMARY

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Rabbit liver microsomal cytochrome P-448 has been highly purified to a specific content of greater than 20 nmoles/mg of protein. Sodium dodecyl sulfate–gel electrophoresis showed the presence of a major polypeptide band with a molecular weight of 51,000 and a faint minor band at 60,000. Purified rabbit cytochrome P-448 differs from cytochrome P-448 purified from rats with respect to its molecular size, its poor reactivity with antibody produced against rat cytochrome P-448, and its relative inactivity for the hydroxylation of several substrates. These results indicate that rabbit cytochrome P-448 and rat cytochrome P-448 are different proteins.

Cytochrome P-450, the terminal oxidase of the microsomal electron transport chain, is induced in microsomes after treatment of animals with drugs or polycyclic hydrocarbons (1). The hemeprotein induced by 3-methylcholanthrene is termed cytochrome P-448, based on an absorption maximum of the reduced CO adduct at 448 nm in contrast to the usual peak at 450 nm.

In rats (2) and in genetically responsive mice strains (3–5), formation of cytochrome P-448 is associated with the induction of benzo[*a*]pyrene hydroxylase activity. In contrast, little or no increase in benzo[*a*]pyrene hydroxylase activity is observed in liver microsomes from rabbits treated with 3-methylcholanthrane (6, 7). This observation raised the possibility that despite its inducibility by 3-methylcholanthrene rabbit cytochrome P-448 may not be identical with rat cytochrome P-448. We have recently reported that partially purified cytochromes P-448 from rabbits and rats have different catalytic activities (8). This report describes the purification of liver microsomal cytochrome P-448 from 3-methylcholanthrene-treated rabbits to apparent homogeneity and demonstrates that rat cytochrome P-448 and rabbit cytochrome P-448 are different hemeproteins.

Table 1 summarizes the results of the procedure used for the purification of cytochrome P-448 from liver microsomes of four 3-methylcholanthrene-treated rabbits. 3-Methylcholanthrene, dissolved in corn oil, was administered subcutaneously to male rabbits (New Zealand strain, 3 kg, Marlin Farms, Hewitt, N. J.) at a dose of 25 mg/kg for 4 days. The animals were killed 24 hr after the last treatment, and liver microsomes were prepared as de-

TABLE 1

Purification of hepatic cytochrome P-448 from 3-methylcholanthrene-treated rabbits

Step	Protein[a]	Cytochrome P-448		
		Total content[b]	Specific content	Yield
	mg	*nmoles*	*nmoles/mg protein*	%
Microsomes	8,950	29,700	3.3 (2.3–3.5)	100
Step I: 40–50% ammonium sulfate fraction	2,620	12,000	4.6 (4.0–5.9)	40
Step II: 42–50% ammonium sulfate fraction	670	3,970	6.0 (4.9–6.0)	14
Step III: calcium phosphate gel	326	2,410	7.3 (6.5–8.1)	8
Step IV: DEAE-cellulose	93	941	10.1 (9.2–10.5)	3
Step V: CM-cellulose	20	336	16.0 (15.2–17.2)[c]	1

[a] Protein concentrations were determined by the method of Lowry *et al.* (9), using bovine serum albumin as the standard.

[b] Cytochrome P-448 concentrations were determined by the method of Omura and Sato (10), using an extinction coefficient of 91 mM^{-1} cm^{-1}.

[c] Based on the amino acid composition, the final specific content was 22–24 nmoles/mg of protein.

scribed previously (11). The initial steps (I, II, and III) were the same as those used for the purification of rat cytochrome P-448 (12) except for the following modification of step III. The concentration of phosphate buffer used to elute the hemeprotein from the calcium phosphate gel was 0.3 M rather than 0.25 M. Subsequent chromatography on DEAE-cellulose (step IV) was essentially the same as described by Levin *et al.* (11), with the exception that the concentration of Emulgen 911 (Kao-Atlas) in all the buffers was increased to 0.2% (w/v). Excess detergent which was not tightly bound to cytochrome P-448 was removed (11) by treating the preparation with Bio Beads SM-2 (Bio-Rad Laboratories).

The partially purified preparation from the DEAE-cellulose column was dialyzed for 3 hr against 5 mM phosphate buffer, pH 7.0, containing 20% glycerol, 0.1 mM EDTA, and 0.1 mM dithiothreitol, and then applied to a column of CM-cellulose (Whatman CM-52, 2.2 × 10 cm) previously equilibrated with 5 mM phosphate buffer, pH 7.0, containing 20% glycerol and 0.1% Emulgen 911. The column was washed sequentially with 50 ml each of 5, 30, and 50 mM phosphate buffers containing 0.1% Emulgen 911 and 20% glycerol. The sample was then eluted from the column with 100 mM phosphate buffer, pH 7.0, containing 20% glycerol and 0.1% Emulgen 911. To remove excess detergent, the hemeprotein was precipitated from the column eluate by adding polyethylene glycol 6000 (J. T. Baker Company) to a final concentration of 15% (w/v), stirring for 20 min, and then centrifuging at 30,000 × *g* for 15 min. The red-brown pellets were dissolved in 0.3 M potassium phosphate buffer, pH 7.4, containing 20% glycerol, 0.1 mM EDTA, and 0.1 mM dithiothreitol. The final Emulgen 911 content was 0.04 mg/nmol of cytochrome P-448. This final preparation was stable for at least 4 months when stored at −20°.

As shown in Table 1, rabbit cytochrome P-448 was purified to a specific content of 15–17 nmoles/mg, based on the protein determination method of Lowry *et al.* (9). However, based on amino acid composition, the final specific content was 22–24 nmoles/mg of protein. The protein content of rat cytochrome P-448 determined by the method of Lowry *et al.* (9) was also greater than that based on its amino acid composition (13). The final preparation was not contaminated with cytochrome b_5, NADPH-cytochrome *c* reductase, or NADH-cytochrome b_5 reductase.

The reduced CO difference spectrum of the purified cytochrome P-488 is shown in Fig. 1. It can be seen that the final prepara-

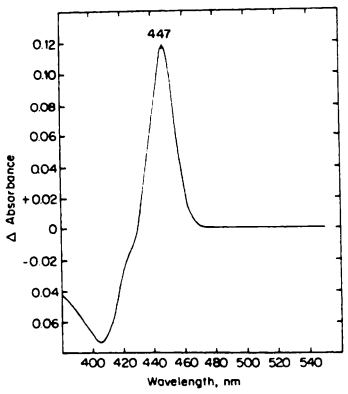

FIG. 1. *Reduced carbon monoxide difference spectrum of highly purified rabbit hepatic cytochrome P-448*

The spectrum was determined at a hemeprotein concentration of 1.3 nmoles/ml.

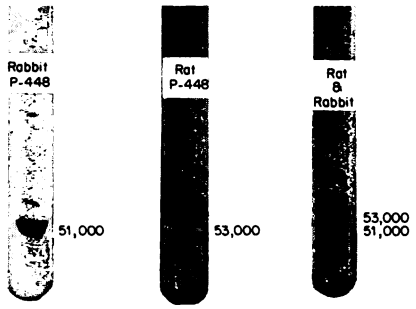

FIG. 2. *Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of highly purified rabbit and rat cytochromes P-448*

The hemeproteins (10 μg) were treated with SDS and (either separately or as a mixture) subjected to polyacrylamide gel electrophoresis by the method of Laemmli (16). The gels were stained with Coomassie blue R250 in the manner described by Fairbanks *et al.* (17).

tion contained an insignificant amount (less than 5%) of cytochrome P-420. The heme concentration of the final preparation was determined as the pyridine hemochromogen as described by Falk (14). The extinction coefficient of the reduced CO complex of the purified rabbit cytochrome P-448 is 89 mM^{-1} cm^{-1}. This is within 5% of the value of 91 mM^{-1} cm^{-1} reported by Imai and Sato (15) for purified rabbit cytochrome and by Ryan *et al.* (13) for purified rat cytochromes P-448 and P-450.

Although not shown, the absolute oxidized spectrum of purified rabbit cytochrome P-448 has absorption maxima at 416, 533, and 566 nm; it also has high-spin characteristics as evidenced by a shoulder at 395 nm and a peak at 644 nm. The absolute reduced spectrum has peaks at 410 and 540 nm. The absolute reduced CO complex shows maxima at 447 and 552 nm and a small shoulder at 423 nm, indicative of the presence of a small amount of cytochrome P-420.

Polyacrylamide gel electrophoresis of the purified rabbit cytochrome P-448 in the presence of sodium dodecyl sulfate (Fig. 2) showed the presence of one major polypeptide having a molecular weight of approximately 51,000 and a very minor component at 60,000. In contrast, rat cytochrome P-448 has a minimum molecular weight of approximately 53,000, and the two hemeproteins can be separated on SDS[1]-gels containing a mixture of the two cytochromes.

Using Ouchterlony double-diffusion analysis, it was found that rabbit cytochrome P-448 cross-reacted very poorly with the antibody prepared against rat cytochrome P-488 (18), thereby demonstrating that rabbit and rat cytochromes P-488 have very different antigenic sites and consequently different structures.

It can be seen in Fig. 3 that purified rat cytochrome P-488 is much more active than rabbit cytochrome P-448 in supporting benzo[*a*]pyrene hydroxylation and benzphetamine *N*-demethylation in the presence of NADPH–cytochrome *c* reductase and phospholipid. Using a fluorometric assay for coumarin hydroxylase activity (24, 25), it was found that purified rabbit cytochrome P-448 could hydroxylate coumarin at a slow rate (16 pmoles/min/nmol of P-448) whereas purified rat cytochrome P-448 was completely inactive.

Using a solubilized rabbit cytochrome P-448 preparation (specific content, 3–4 nmoles/mg of protein), Philpot and Bend (26) recently suggested that the low rate of benzo[*a*]pyrene hydroxylation catalyzed by rabbit cytochrome P-448 may be due to the presence of an endogenous inhibitor in

[1] The abbreviation used is: SDS, sodium dodecyl sulfate.

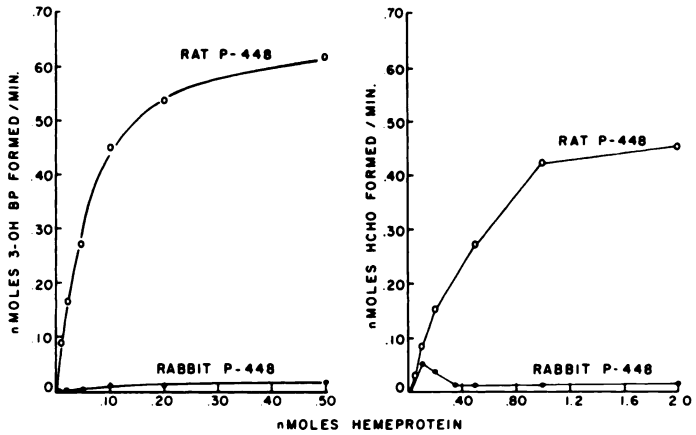

FIG. 3. *Comparison of benzo[a]pyrene hydroxylase (left) and benzphetamine N-demethylase (right) activities of purified rabbit and rat cytochromes P-448*

The hydroxylation of benzo[a]pyrene (BP) by the reconstituted system was determined fluorometrically according to Nebert and Gelboin (19) as described previously (20). The reactions were carried out at 37° for 5 min in the presence of 125 units of NADPH–cytochrome *c* reductase, 0.1 mg of lipid, and the indicated amounts of hemeprotein. The *N*-demethylation of [*N*-methyl-^{14}C]benzphetamine was measured by quantitating the formation of [^{14}C]formaldehyde (21, 22). Reactions were performed at 37° for 30 min in the presence of 250 units of NADPH–cytochrome *c* reductase, 0.1 mg of lipid, and all other required components (20). The NADPH–cytochrome *c* reductase (23) and lipid (20) fractions were prepared as described previously. One unit of NADPH–cytochrome *c* reductase equals 1 nmole of cytochrome *c* reduced per minute.

the preparation. Our studies indicate that this difference in catalytic activity between rat cytochrome P-448 and rabbit cytochrome P-448 is due to the fact that they are different hemeproteins, a conclusion based on the following evidence: (a) rabbit and rat cytochromes P-448 have different molecular weights as determined by SDS-gel electrophoresis, (b) rabbit cytochrome P-448 reacted poorly with antibody against rat cytochrome P-448 using Ouchterlony double-diffusion analysis, and (c) hydroxylation of benzo[a]pyrene supported by rabbit P-448 was more susceptible to inhibition by 7,8-benzoflavone and diethylaminoethyl diphenylpropyl acetate (SKF 525-A) than hydroxylation catalyzed by rat cytochrome P-448 (8).

Thus the multiplicity of liver microsomal cytochrome P-450 can be observed in at least three different situations *in vivo*: (a) a single animal, either untreated or treated with an inducer, has multiple forms of liver microsomal cytochrome P-450 (27–29); (b) different forms of cytochromes P-450 are evident when a particular species of animal is treated with different inducers (e.g., cytochrome P-450 in phenobarbital-treated rats vs. cytochrome P-448 in 3-methylcholanthrene-treated rats (27); and (c) different forms of cytochrome P-450 are present in different species of animals treated with the same inducer (as reported in this paper for 3-methylcholanthrene-treated rats and rabbits). In conclusion, the existence of multiple forms of cytochromes P-450 may explain the species, strain, age, tissue, and sex differences observed in drug metabolism.

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