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ROLE OF ISOSAFROLE AS COMPLEXING AGENT AND INDUCER of P-450_{LM4} IN RABBIT LIVER MICROSOMES

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SUMMARY: Isosafrole serves as an excellent inducing agent in rabbits for P-450_{LM4}, the same isozyme as that which is induced by 3-methylcholanthrene and 5,6-benzoflavone. Since the isosafrole adduct formed with isozyme 4 is unstable, the uncomplexed cytochrome may be purified in very good yield (over 30%, based on total P-450) from liver microsomes of animals given this agent. Isosafrole forms adducts with both isozyme 2 (the phenobarbital-inducible form of P-450) and isozyme 4 in the reconstituted system containing NADPH-cytochrome P-450 reductase, phosphatidylcholine, and NADPH under aerobic conditions, but with the latter cytochrome the reaction has a lower K_m and maximal rate.

The addition of synergists to insecticides has a sparing action, so that lower doses of the insecticide then have the same effect (1). Many synergists were found to exhibit substrate binding spectra upon addition to hepatic microsomes (2), and it was proposed that the methylenedioxyphenyl synergists may act by affecting the integrity of the electron transfer chains involved in mixed function oxidation reactions (3). Philpot and Hodgson (4) reported that an ethyl isocyanide-like difference spectrum with absorption maxima at 455 and 428 nm was produced by the addition of piperonyl butoxide and a reduced pyridine nucleotide to mouse liver microsomes, and Franklin (5) demonstrated that various methylenedioxyphenyl compounds (piperonyl butoxide, methylenedioxybenzene, safrole, and isosafrole) are metabolized by rat liver microsomes in the presence of oxygen and NADPH (but not NADH) to give products which exhibit an isocyanide-like absorption spectrum with the reduced cytochrome P-450. Safrole and isosafrole cause the induction of a unique hepatic microsomal hemoprotein in the rat (6) which is present as a stable complex (7); this form of cytochrome P-450 has been purified to electrophoretic homogeneity as the isosafrole metabolite complex (8,9). The dissociation of the complex by added substrates to generate free cytochrome P-450 has been described (10,11), and a linear relationship has been observed between the amount of complex decomposed and the ability of the

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cytochrome P-450 liberated to form the carbon monoxide complex or catalyze the hydroxylation of biphenyl (12).

As reported in the present paper, quite different results were obtained upon the administration of isosafrole to rabbits. This compound serves as an inducer of the same isozyme, P-450$_{LM_4}$,[1] as do 3-methylcholanthrene and 5,6-benzoflavone, rather than a unique cytochrome. Furthermore, the isosafrole adduct is unstable and upon purification the uncomplexed cytochrome is isolated.

MATERIALS AND METHODS

P-450$_{LM_2}$ (13,14) and NADPH-cytochrome P-450 reductase (15,16) were isolated from liver microsomes of phenobarbital-induced rabbits. P-450$_{LM_4}$ was isolated from liver microsomes of rabbits treated with phenobarbital, which does not induce this cytochrome, or with 5,6-benzoflavone or isosafrole (17), which serve as inducers, according to published procedures (13,-14). For reconstitution of the enzyme system, 1.0 nmol of P-450, 1.0 nmol of reductase, and 50 µg of sonicated dilauroyl-GPC were mixed, and after 2 min the mixture was diluted to a volume of 1.0 ml by the addition of potassium phosphate buffer (pH 7.4), glycerol, and EDTA at final concentrations of 50 mM, 10%, and 0.1 mM, respectively. For complex formation of P-450 with isosafrole, various amounts of this compound (in 5 µl of dimethylformamide) were added to the reconstituted enzyme system at final concentrations from 1.5 to 80 µM. NADPH was the last addition at a final concentration of 150 µM, both for spectral studies with isosafrole and for determination of the rate of deethylation of ethoxycoumarin. Spectra were recorded at room temperature with use of a Cary 219 recording spectrophotometer with repetitive scanning from 500 to 400 nm or an Aminco recording spectrophotometer at 455 or 458 nm (for P-450$_{LM_4}$ and $_{LM_2}$, respectively), and 490 nm for the reference wavelength.

RESULTS AND DISCUSSION

As already indicated, treatment of rats with isosafrole results in the formation of an isozyme of hepatic microsomal cytochrome P-450 which is unique for this inducer (8,9). In contrast, the administration of this compound to rabbits causes extensive induction of P-450$_{LM_4}$, an isozyme known to be induced by carcinogens and related compounds. The pyrophosphate-washed hepatic microsomes from isosafrole-treated rabbits have as much as 21% of the total P-450 present as a complex with an absorbance maximum at 455 nm, and only about 10% of the total P-450 is observable as the reduced carbonyl complex. However, solubilization of the microsomes with either sodium cholate or the nonionic detergent Tergitol NP-10 results in complete dissociation of the isosafrole complex, and all of the cytochrome is then detectable as the reduced carbonyl complex. With the use of procedures developed in this laboratory (14), the enzyme was purified to electrophoretic homogeneity (Table I) with an overall

[1]The abbreviations used are: P-450$_{LM}$, liver microsomal cytochrome P-450; and dilauroyl-GPC, dilauroylglyceryl-3-phosphorylcholine. The isozymes of P-450$_{LM}$ are numbered according to their relative mobilities when submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis; P-450$_{LM_2}$ and $_{LM_4}$ are induced by phenobarbital and 5,6-benzoflavone, respectively.

TABLE I

Purification of P-450_{LM_4} from Liver Microsomes of Isosafrole-treated Rabbits

Preparation	Protein (mg)	Cytochrome P-450 content (nmol/mg protein)	Yield[a] %
Pyrophosphate-washed microsomes	1530	3.3	100
Polyethylene glycol 6000 precipitate (8-15%)	809	5.0	80
DEAE-cellulose column eluate	244	8.1	39
Hydroxylapatite column eluate	88.7	18.0	32

[a]The yield is based on the total amount of P-450 in the microsomes.

yield greater than 30% of the total P-450 present in the microsomes. No 455 nm complex was observed following solubilization, in contrast to the rat liver enzyme, which was purified with a portion of the cytochrome in the complexed state (8,9). The primary structure of isozyme 4 from isosafrole-treated rabbits is apparently identical with that of the enzyme isolated from untreated and phenobarbital- or 5,6-benzoflavone-treated animals as determined by peptide mapping, immunological reactivity, and N-terminal sequencing (results not shown).

When reconstituted with dilauroyl-GPC, NADPH-cytochrome P-450 reductase, and NADPH under aerobic conditions, both isozyme 2 and isozyme 4 form a metabolic complex with isosafrole, as shown in Table II. Isozyme 4 from the three sources has the same spectrum for the isosafrole complex, and gives K_m values for isosafrole and V_{max} values for complex formation which are not significantly different. The wavelength maxima of the isosafrole complexes of the two cytochromes are red-shifted slightly from the maxima of the corresponding reduced-carbonyl complexes, thus suggesting that CO and isosafrole serve as similar ligands for the reduced heme iron. While isozyme 2 forms the isosafrole

TABLE II

Formation of Isosafrole Complexes with Purified Isozymes 2 and 4

Agent administered	Isozyme	Complex formation[a] λ_{max} (nm)	K_m (µM)	V_{max} (nmol/min/nmol P-450)
Phenobarbital	2	458	14	0.21
Phenobarbital	4	455	3.0	0.06
Isosafrole	4	455	3.2	0.10
5,6-Benzoflavone	4	455	3.0	0.07

[a]The K_m and V_{max} were determined from double reciprocal plots of the initial rate of complex formation with isosafrole concentrations ranging from 1.0 to 80 µM. An extinction coefficient of 75 $mM^{-1}cm^{-1}$ was used for the complex, as described in the text.

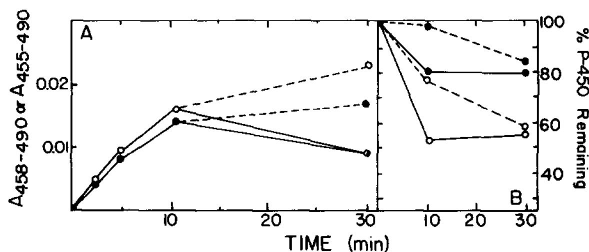

Fig. 1. Formation and decay of isosafrole metabolic complexes with purified isozymes 2 (o) and 4 (•). The isosafrole complex was measured by difference spectroscopy as described in Methods following the addition of dithionite, and the carbon monoxide complex was measured after the cell had been bubbled with CO, followed by reduction with dithionite. Panel A shows the levels of the isosafrole complexes at various times following initiation of the reaction by the addition of NADPH (150 μM) at zero time in the presence of 80 μM isosafrole (solid curves). In other experiments, a second equal addition of NADPH was made at 10 min (dashed lines). Panel B shows the amount of P-450 remaining in reaction mixtures like those in Panel A but with NADPH added at zero time only and with the inclusion of controls containing no isosafrole (dashed lines).

complex at a greater rate than isozyme 4, the K_m of isosafrole for the latter protein is significantly lower. Thus, even though isozyme 4 has a lower rate of complex formation at saturating levels of isosafrole, it may be the enzyme primarily responsible for the metabolism of this compound at low concentrations. It should also be noted that isozyme 4 is the major form of P-450 present in the hepatic reticulum of normal rabbits, whereas isozyme 2 is present at insignificant levels unless the animals have been exposed to phenobarbital or related inducers.

The formation of the 455 nm complex with isosafrole results in a decrease in the amount of P-450 detectable by combination with carbon monoxide (Fig. 1). An extinction coefficient of 75 $mM^{-1}cm^{-1}$ was obtained for the isosafrole metabolite complex from the direct relationship between the increase in absorbance of the 455 nm complex and the decrease at 447 nm of the CO-complex (results not shown), in agreement with the value reported by others (5). Only about 30% of either isozyme 2 or 4 is present as the complex after 10 min in the presence of NADPH. At a constant concentration of P-450, the level of the complex was not increased by increasing the concentration of any of the other components of the reconstituted system. When the reaction mixtures were supplemented with a second addition of NADPH after 10 min, there is a slight increase in the level of complex formed with both LM_2 and LM_4.

Following the depletion of the reducing equivalents in the system at about 10 min, the absorbance of the complex for both LM_2 and LM_4 decreases. Approximately 50% of the LM_2 complex and 20 to 30% of the LM_4 complex is lost by 30 min. After the elimination of isosafrole and free metabolites by dialysis (or in experiments not shown, by treatment with Amberlite XAD 2), a large percent-

TABLE III

Effect of 455-nm Complex on Enzyme Activities[a]

Components	P-450 remaining after dialysis (nmol)	Complex formation K_m (μM)	Complex formation V_{max} (nmol/min/nmol P-450)	Ethoxycoumarin deethylation (nmol/min/nmol P-450)
Isozyme 2				
Complete	0.78	15	0.24	11.3
-Isosafrole	0.95	25	0.37	11.2
-NADPH	1.05	14	0.23	9.3
Isozyme 4				
Complete	0.35	2.0	0.09	1.6
-Isosafrole	0.53	2.9	0.13	1.9
-NADPH	0.72	2.8	0.11	1.6

[a]Isozyme 2 or 4 was incubated for 30 min in the reconstituted system containing isosafrole or with isosafrole or NADPH omitted as indicated. Each mixture was then dialyzed, and complex formation with added isosafrole and the deethylation of added ethoxycoumarin were determined. The deethylation of ethoxycoumarin was determined by the fluorometric method of Greenlee and Poland (18). The data are expressed per nmol of P-450 remaining as measured by the CO difference spectrum.

age of both isozyme 2 or 4 is modified so that the CO-complex is not observable (Table III); the enzyme was not detected as the inactive P-420 form. The nature of this modification is not understood at the present time. The LM_2 or LM_4 which is detectable as the CO-complex after dialysis exhibit similar rates of complex formation and ethoxycoumarin deethylation as those obtained in the initial reaction mixture (Table III).

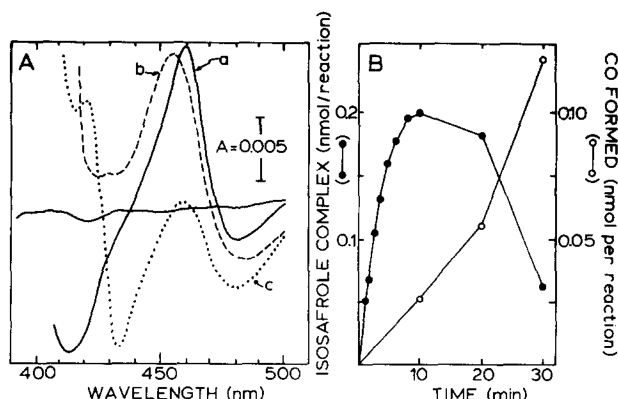

Fig. 2. Formation of carbon monoxide complex of isozyme 2 associated with dissociation of the isosafrole metabolite complex following NADPH consumption. In the experiment summarized in Panel A a reaction mixture containing the reconstituted system (without isosafrole) was divided equally into two cuvettes, and the baseline was recorded. Isosafrole was added to the sample cuvette and NADPH was added to both cuvettes, and spectrum a was recorded after 10 min. The complex was largely dissociated by 30 min, at which time dithionite was added to both cuvettes, and spectrum b was recorded; 100 μg of hemoglobin were then added to each cuvette, and spectrum c was obtained. Panel B shows the formation and decay of the isosafrole complex and the concomitant liberation of carbon monoxide. The amount of carbon monoxide was determined by the absorbance difference, 451-490 nm; CO was displaced by the addition of hemoglobin.

Following the decomposition of the isosafrole complex after the NADPH present in the reaction mixture was consumed, as illustrated in Fig. 1, the reduction of the system with sodium dithionite results in a blue shift of the spectrum to the absorbance maximum of the reduced CO complex. The results of such an experiment are shown in Fig. 2. The blue shift of the spectral maximum suggests that CO formed by decomposition of the isosafrole complex is then bound to the P-450. That CO was formed was further indicated by an experiment in which hemoglobin was added to the reaction mixture following dithionite reduction; the presence of the peak at 420 nm due to the formation of carboxyhemoglobin (spectrum c in Fig. 2) confirmed the presence of CO. Furthermore, the amount of carbon monoxide formed is directly related to the amount of the 455-nm complex displaced, as shown in Fig. 2B. Carbon monoxide has been identified as a metabolite of methylenedioxy compounds which form low levels of the 455 nm complex in rat liver microsomes (19). The K_i for CO in the inhibition of isosafrole complex formation is about 20 μM. Thus, the levels of CO produced during decomposition of the isosafrole complex would probably not result in significant inhibition.

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