Isosafrole-induced Cytochrome P₄-450 in DBA/2N Mouse Liver

CHARACTERIZATION AND GENETIC CONTROL OF INDUCTION

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Mouse "cytochrome P₄-450" is defined as that form of isosafrole-induced P-450 in DBA/2N liver most specifically correlated with isosafrole metabolism. Isosafrole pretreatment does not induce aryl hydrocarbon hydroxylase activity ("cytochrome P₂-450") in C57BL/6N or DBA/2N mice, induces acetanilide 4-hydroxylase activity ("cytochrome P₃-450") more than 3-fold in C57BL/6N but not in DBA/2N mice, and induces isosafrole metabolite formation more than 3-fold in both C57BL/6N and DBA/2N mice. P₂-450 was, therefore, purified from isosafrole-treated DBA/2N liver microsomes having negligible amounts of contaminating P₁-450 and P₂-450. The apparent molecular weight of P₂-450 is 55,000, and the protein appears homogeneous on sodium dodecyl sulfate-polyacrylamide gels. The Soret peak of the reduced purified cytochrome CO complex is 448 nm. Purified P₂-450, reconstituted in vitro, metabolizes acetanilide poorly and benz[a]pyrene hardly at all. Anti-(P₂-450) inhibits (90 to 100%) liver microsomal isosafrole metabolite formation, yet has no effect on aryl hydrocarbon hydroxylase, acetanilide 4-hydroxylase, biphenyl 2- or 4-hydroxylase, or 7-ethoxyxocumarin O-deethylation activities. 3-Methylencholanthrene induces anti-(P₂-450)-precipitable protein about 12-fold in C57BL/6N and 2-fold in DBA/2N liver; 2,3,7,8-tetrachlorodibenzop-dioxin (10 μg/kg), about 12-fold in both C57BL/6N and DBA/2N liver; isosafrole, more than 3-fold in both C57BL/6N and DBA/2N. Benzo[a]pyrene at maximal doses induces anti-(P₂-450)-precipitable protein in C57BL/6N liver no more than 2-fold, yet is known to be a highly potent inducer of P₁-450 mRNA in C57BL/6N liver.

The sensitivity of the P₂-450 induction process to isosafrole is inherited as an autosomal additive trait; studies of offspring from the C57BL/6N(DBA/N)F₁ × DBA/2N backcross confirm involvement of the Ah locus or a closely segregating gene. In contrast, among crosses between DBA/2N and C57BL/6N, sensitivity of the P₁-450 and P₂-450 induction process to 3-methylcholanthrene or 2,3,7,8-tetrachlorodibenzop-p-dioxin is inherited as an autosomal dominant trait. These data suggest that, although P₁-450, P₂-450, and P₃-450 proteins are controlled by the Ah locus, either a P-450 protein polymorphism exists between C57BL/6N and DBA/2N mice or subtle differences may exist in the interaction of various inducers with Ah receptor.

The biochemical purification and characterization of a growing number of distinct cytochrome P₄-50 proteins has received increasing attention in recent years. More than 300 foreign chemicals, steroids, and peptide hormones are known to enhance P-450-mediated monooxygenase activities (reviewed in Refs. 4 and 5), suggesting the induction of one or more P-450 proteins in each instance, and the list grows larger each year.

To understand the genetic mechanisms by which a particular chemical is able to induce a particular P-450 protein has been the central goal of this laboratory. Most of our work has involved the expression of mouse P-450, controlled by the Ah receptor (6-8). These studies have always taken advantage of the fact that the B6 mouse (Ah₂/Ah₂) has a large amount of the high affinity receptor and the D2 mouse (Ah²/Ah²) has a poor affinity receptor. Following 3-methylcholanthrene treatment, P₁-450 is thus highly induced in B6 mouse liver and negligibly induced in D2 mouse liver. P₂-450 induction by 3-methylcholanthrene is inherited in the B6D2F₁, heterozygote (Ah₂/Ah²) as an autosomal dominant trait (9).

Isosafrole is a naturally occurring plant constituent (10). Studying Ah²/Ah² and Ah²/Ah² mice, Fennell et al. (11) concluded that isosafrole induces a form of P-450 specific for...
isosafrole metabolism and that this form is not identical to the major phenobarbital- or 3-methylcholanthrene-inducible forms of P-450. Subsequently, isosafrole-induced P-450 was purified from rat liver microsomes (12, 13). From these studies it was apparent that this isosafrole-induced P-450 is similar in many ways to the major rat 3-methylcholanthrene-inducible P-450 yet did not metabolize benzo(α)pyrene. These valuable studies (3, 6-8, 12, 13) were each performed with an antibody that was not claimed to be monospecific; despite this shortcoming many important conclusions have been made.

Whereas 3-methylcholanthrene induces P-450 in B6 but not D2 liver, isosafrole induces a form of P-450 almost equally well in both B6 and D2 liver (1, 11). In this report we show that the isosafrole-induced form in many ways is not identical to the major rat 3-methylcholanthrene-inducible P-450, and show that this isosafrole-induced P-450 is not monospecific; despite this shortcoming many important conclusions have been made.

EXPERIMENTAL PROCEDURES

Materials—Benzo(α)pyrene, 3-methylcholanthrene, benz[a]anthracene, dithiothreitol, dilauroylphosphatidylcholine, 7-ethoxycoumarin, dexamethasone, NADPH, and NADH were purchased from Sigma; 2-hydroxybiphenyl, 4-hydroxybiphenyl, 2-methylnitrosoaniline, acetanilide, and 4-hydroxyacetanilide from Aldrich; isosafrole from Eastman Kodak Company (Rochester, NY); sodium cholate and BSA (tracendase, Wistetgen TM-2; N-tetradecyl-N,N-dimethyl-3-ammonio-1-propane sulfonate) from Calbiochem-Behring; hydroxylapatite (Bio-Gel HT) from Bio-Rad; sodium phenobarbital from Merck; NaB[3H]4 (7.0 Ci/mmol) from Amersham Corp.; acetanilide uniformly labeled with 14C in the ring (10.5 mCi/mmol) from California Bionuclear Corporation (Sun Valley, CA); ellipticine from the National Cancer Institute, Chemical Center (Bethesda, MD); P-2-450 (specific activity of ~15.0 mmol/mg of protein) was obtained from Bio-Rad. Immunoglobulin G fractions were prepared by ammonium sulfate fractionation. After dialysis the fractions were dissolved in 100 mM potassium phosphate buffer (pH 7.5). These fractions were used for inhibition of microsomal monooxygenase activities in vitro (3) and determination of tritiated anti-(P-450)-precipitable protein from cholate-solubilized microsomal membranes that had been previously labeled with NaB[3H]4 in the presence of 1 mM pyridoxal phosphate (20, 21), by the methods cited. The values of anti-(P-450)-precipitable protein expressed as: (disintegrations min-1 per mg of microsomal protein)/(disintegrations min-1 per mg of total microsomal protein).

Fig. 1. Elution profile from an octylidine-Sepharose 4B column. The solubilized microsomes were applied to the column, and P-450 was eluted by the buffer conditions described under "Experimental Procedures." Fractions 1 to 49 were eluted with 0.05% Emulgen 913; fractions 50 to 67 were eluted with 0.20% Emulgen 913. The inset represents NaDodSO4-polyacrylamide gel electrophoresis analysis (16, 19) of several selected fractions.
Results

The P2-450 Protein—As detailed under "Experimental Procedures" and in Fig. 1, an electrophoretically homogeneous protein was purified from isosafrole-treated D2 liver microsomes. The Soret peak of the reduced cytochrome-CO complex was 449 nm in microsomes (Fig. 2), but the Soret peak of the purified P2-450-CO complex was 448 nm. The apparent molecular weight was 55,000 (Fig. 3). By means of Ouchterlony double diffusion plates, fusion of a major precipitin line between neighboring wells (Fig. 4) indicates immunologic homology of the anti-(P2-450)-precipitable protein in isosafrole-treated B6 and D2 and control D2 liver microsomes. An increased intensity of the precipitin line following isosafrole treatment is consistent with relatively equal induction of anti-

(P2-450)-precipitable material in both D2 and B6 mice. A possible minor precipitin line nearer to well I than the major line (Fig. 4) suggests that the antiserum may recognize at least a second microsomal protein; we are not claiming, however, that anti-(P2-450) is monospecific. There are clearly no indications that isosafrole is not a good inducer of P1-450 in either D2 or B6 mice and does not induce P3-450 in D2 mice.
that the metabolite forms a complex with the induced protein.

3-Methylcholanthrene, on the other hand, induced both aryl hydrocarbon hydroxylase and acetanilide 4-hydroxylase activity more than 6-fold in B6 mice and not at all in D2 mice. These data have been reported previously (3, 27).

The P2-450-reconstituted activities of aryl hydrocarbon hydroxylase and acetanilide 4-hydroxylase (Table I) are about one-thirtieth and one-tenth, respectively, of these activities in 3-methylcholanthrene-induced B2 liver intact microsomes. The rate of benzo[a]pyrene metabolism by purified P1-450 reconstituted in vitro (3) was 2.95 nmol/min/nmol of P-450, as compared with the P2-450 value of 0.14 in Table I. The rate of acetanilide 4-hydroxylation by purified P2-450 reconstituted in vitro (3) was 32 nmol/min/nmol of P-450, as compared with the P2-450 value of 1.52 in Table I. These findings suggest that, whereas there exist overlapping substrate specificities, neither benzo[a]pyrene nor acetanilide is a particularly good substrate for P2-450. Further, the results in Table I show that isosafrole-induced P2-450 in D2 mice is different from 3-methylcholanthrene-induced P1-450 or P3-450 in B6 mice.

Isosafrole induced total P-450 content in D2 liver (Table II), whereas 3-methylcholanthrene had no effect in D2 liver. In B6 liver, 3-methylcholanthrene induced total P-450 content better than isosafrole treatment. It is known (12, 13, 22) that isosafrole is metabolized by isosafrole-induced P450 and that the metabolite forms a complex with the induced protein.

The rate of benzo[a]pyrene metabolism by purified P1-450 reconstituted in vitro (3) was 2.95 nmol/min/nmol of P-450, as compared with the P2-450 value of 0.14 in Table I. The rate of acetanilide 4-hydroxylation by purified P2-450 reconstituted in vitro (3) was 32 nmol/min/nmol of P-450, as compared with the P2-450 value of 1.52 in Table I. These findings suggest that, whereas there exist overlapping substrate specificities, neither benzo[a]pyrene nor acetanilide is a particularly good substrate for P2-450.

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**TABLE I**

_Aryl hydrocarbon hydroxylase and acetanilide 4-hydroxylase activity in intact liver microsomes and with purified P1-450 reconstituted in vitro._

Values represent triplicate determinations on liver microsomes from six mice or, with the reconstituted assays _in vitro_, the average of two experiments in duplicate. Specific activities are expressed as nanomoles of product formed per min per nmol of P-450. Products are phenolic benzo[a]pyrene metabolites and 4-hydroxycetanilide, respectively. Further details are described under "Experimental Procedures."

| Fraction          | _In vivo_ treatment | Aryl hydrocarbon hydroxylase | Acetanilide 4-hydroxylase |
|-------------------|---------------------|-------------------------------|---------------------------|
|                   |                     | D2 | B6 | D2 | B6             |
| Intact microsomes | Control             | 0.70 | 0.75 | 2.66 | 2.68          |
|                   | Isosafrole          | 0.66 | 0.78 | 2.50 | 8.20          |
|                   | 3-Methylcholanthrene | 0.60 | 4.87 | 2.72 | 17.0          |
| Reconstituted     | +P1-450             | 0.14 |     | 1.52 |               |
|                   | -P2-450             | <0.01 |     | <0.01 |               |

**Formation of the P-450 complex with isosafrole metabolite and the effect of anti-(P2-450) on this formation**

Values represent at least two determinations on liver microsomes pooled from six mice. Each determination of specific activity was also performed in duplicate.

| Inbred strain | Treatment _in vivo_ | Total P-450 content | Formation of isosafrole metabolite with P-450 | Specific isosafrole activity (rate of formation of isosafrole complex with P-450) |
|---------------|---------------------|---------------------|-----------------------------------------------|--------------------------------------------------------------------------------|
|               |                     |                     | _in vivo_ | _in vitro_ | -Anti-(P2-450) | +Anti-(P2-450) |
| D2            | Control             | 1.02                | 0.10    | 0.049     | 0.040       |
|               | Isosafrole          | 1.51                | 0.32    | 0.080     | <0.001      |
|               | 3-Methylcholanthrene | 1.04              | 0.11    | 0.047     | 0.040       |
| B6            | Control             | 0.77                | 0.12    | 0.066     | 0.060       |
|               | Isosafrole          | 1.58                | 0.42    | 0.107     | 0.010       |
|               | 3-Methylcholanthrene | 2.04              | 1.1     | 0.193     | 0.056       |
|               | Phenobarbital       | 2.42                | 0.60    | 0.109     | 0.105       |
Liver microsomes from isosafrole-treated B6 and D2 were examined as freshly prepared intact samples (in duplicate) for each value shown. Inducer treatment included procedures. A, radioactivity measured as a single 55,000-dallon peak from control, 3-methylcholanthrene (MeChol)-treated, isosafrole-treated, and control B6 (top) and D2 (bottom) mice. The immune serum (immunoglobulin G) in milligrams of protein is depicted on the abscissa. The starting liver microsomes (10 mg of protein/ml) were dissolved in 100 mM potassium phosphate (pH 7.5) containing 200 mM KCl, 20% glycerol, and 2% sodium cholate. Following centrifugation at 105,000 × g for 60 min, aliquots of the supernatant (40 μg) were used for precipitation by the immune serum. The incubation was carried out overnight at 4 °C. Immunoprecipitates were washed twice with 100 mM potassium phosphate buffer (pH 7.5) containing 200 mM KCl, 1% sodium cholate, and 0.25% SB10, and then once with water to remove potassium ions.

P<sub>2</sub>-450 almost as well. These data are consistent with the finding (30) that D2 mice have a poor affinity Ah receptor that can be overwhelmed by sufficient amounts of TCDD; when this occurs, the TCDD-receptor complex enters the nucleus and elicits its effect of P<sub>1</sub>-450 mRNA induction (30). The results with 3-methylcholanthrene and TCDD in B6 and D2 mice in Fig. 7 thus suggest that induction of the anti-(P<sub>2</sub>-450)-precipitable material might be controlled by the Ah receptor (5, 6).

Benzo[a]anthracene induced the immunoprecipitate only about 2-fold in B6 and negligibly in D2 mice (Fig. 7). It is known (17) that the dose of benzo[a]anthracene used induces P<sub>1</sub>-450 mRNA very effectively in B6 but not D2 mice. Ellipticine behaved like isosafrole by inducing anti-(P<sub>2</sub>-450)-precipitable protein equally well in B6 and D2 mice. A single 55,000-dallon peak from control, 3-methylcholanthrene (MeChol)-treated, isosafrole-treated, and phenobarbital-treated B6 (left) and D2 (right) mice. B, amount of [H]protein precipitated by anti-(P<sub>2</sub>-450) from liver microsomes of B6 and D2 mice that had received treatment with nine various chemicals known to induce one or more forms of P<sub>4</sub>-450. B6 and D2 mice were also treated with three other derivatives of the p,p'-DDT isocarboxide, p,p'-DDE, Dicofol, and chlorobenzylate; the results were not significantly different from that seen with p,p'-DDE, dexamethasone, pregnenolone 16α-carbonitrile, or phenobarbital treatment. The amount of immunoprecipitated P<sub>4</sub>-450 protein labeled with tritium was calculated on the basis of disintegrations per min per mg of protein in the cholate-solubilized fraction. Individual samples varied less than 10%. Further experimental details are described under "Experimental Procedures." the legend to Fig. 6, and in Refs. 3 and 20.

2 Isosafrole, benzo[a]anthracene, and ellipticine displace 67, 61, and 40%, respectively, of [H]TCDD from the Ah receptor (31), when these test compounds are present in 100-fold excess, compared with the radioligand concentration of 10 nM; in the same experiments, 3-methylcholanthrene and nonlabeled TCDD displace the radioligand 95 and 100%, respectively (31).
nontprecipitate in either B6 or D2 mice.

Induction of Anti-(P2-450)-precipitable Material by Isosafrole—Because aryl hydrocarbon hydroxylase activity is not induced by isosafrole, we cannot conveniently study aryl hydrocarbon hydroxylase induction in this experiment.

Association of the Ah Locus with Induction of the Immuno-
precipitate by Isosafrole—An intermediate dose of isosafrole (15 mg/kg) was used to demonstrate that the additive inheritance of P2-450 induction follows Mendelian genetics and correlates well with the Ah phenotype (Fig. 9). The B6D2F1, which is intermediate between B6 and D2 parent, about one-half appeared to be similar to B6 and about one-half similar to the F1. From offspring of the B6D2F1, about one-half appeared to be similar to the F1, and about one-half similar to the D2 parent. Although these individual mouse values are consistent with single-gene inheritance of an additive trait, there is sufficient scatter of points to suggest more than one gene being involved.

In Fig. 9 (far right), eleven offspring from the B6D2F1, about one-half appeared to be similar to the B6 and about one-half similar to the F1. From offspring of the B6D2F1, about one-half appeared to be similar to the F1, and about one-half similar to the D2 parent. Although these individual mouse values are consistent with single-gene inheritance of an additive trait, there is sufficient scatter of points to suggest more than one gene being involved.

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Discussion

In this report we have shown the isolation and characterization of a new mouse P-450 protein called P2-450. We have demonstrated that P2-450 induction by classical P-450 inducers, and genetic expression of its induction among the appropriate crosses between B6 and D2 mice. An antibody’s "lack of induction of catalytic activity" need not always be correlated with the antibody’s "lack of immu...
noprecipitation.” It is also possible that an antibody might precipitate the protein without blocking its catalytic activity. P2-450 seems highly specific for isosafrole metabolite formation and metabolizes acetanilide and benzo[a]pyrene very poorly. Anti-(P2-450) blocks the isosafrole metabolite formation virtually 100% and has no effect on acetanilide 4-hydroxylaty, benzo[a]pyrene hydroxylation, biphenyl 2- or 4-hydroxylation, or 3-ethoxycoumarin O-de-ethylation.

The additive inheritance of the induction of anti-(P2-450)-precipitable protein between B6 and D2 mice might be explained on the basis of a P-450 polymorphism between the two inbred strains. Complete nucleotide sequencing of the mRNA from both strains will rule this possibility in or out.

The variable types of inheritance and “induction responses” of P1-450, P2-450, and P3-450 by several polycyclic aromatic compounds may be difficult to resolve by postulating a single species of Ah receptor. It, therefore, appears that there exist at least four groups of polycyclic aromatic compounds which interact in slightly different ways with the Ah receptor to induce P1-450, P2-450, and P3-450 differentially. First, 3-methylcholanthrene, when given at the highest experimentally possible doses, induces P1-450 in B6 but negligibly in D2 mice (36). 3-Methylcholanthrene also induces the P2-450 immunoprecipitate and P2-450 very well in B6 mice but not D2 mice. 3-Naphthalolavone (57) presumably belongs to this 3-methylcholanthrene class. Second, TCDD at low doses induces P1-450 in B6 but not D2 mice yet at high doses induces P1-450 equally well in both B6 and D2 mice (30, 36). TCDD also has the same effect on P2-450 (Fig. 7B) and P2-450 (2) induction. Third, isosafrole at low doses induces the anti-(P2-450)-precipitable protein in B6 but not D2 mice and at high doses induces the immunoprecipitate equally well in both B6 and D2 mice. However, isosafrole at high doses does not induce P1-450 and induces acetanilide 4-hydroxylase activity in B6 but not D2 mice. Fourth, benzo[a]anthracene is an excellent inducer of P2-450 mRNA in B6 but not D2 mice (17) but induces the P2-450 immunoprecipitate very poorly in B6 mice.

In terms of classical receptor studies, when an inducer-receptor complex forms and interacts with an intranuclear target, one specific response is elicited. If a different response is elicited with a second inducer, this suggests a distinctly different inducer-receptor complex. Therefore, it seems impossible that any two of the above-mentioned four classes of compounds are acting precisely in the same manner. One possible explanation is that the Ah receptor population is heterogeneous (31). The other possibility is that a single type of Ah receptor molecule exists but that each of several inducers heterostrically produces a somewhat different structure of the inducer-receptor complex, thereby exerting different signals during the activation of the three P-450 genes, plus one or more UDP-glucuronosyltransferase(s). Further studies with these four (or more) proteins, and especially their corresponding genes, should shed new light on this perplexing problem.

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