 Catalysis of Divergent Pathways of 2-Acetylaminofluorene Metabolism by Multiple Forms of Cytochrome P-450

Eric F. Johnson,1 David S. Levitt, Ursula Muller-Eberhard,2 and Snorri S. Thorgeirsson

ABSTRACT

Four highly purified forms of rabbit hepatic, microsomal cytochrome P-450 catalyze the N- and ring-hydroxylation of 2-acetylaminofluorene (AAF) at different rates. Form 4, the major form of the cytochrome induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin in adult rabbit liver, catalyzed the N-hydroxylation of AAF more rapidly than did the other three forms. N-Hydroxy-2-acetylaminofluorene accounted for 70% of the metabolites formed by the action of this cytochrome. Form 6, the major form of the cytochrome induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin in neonate rabbit liver, and Form 3, a constitutive form of the cytochrome, both metabolized AAF at one-half the rate observed for Form 4. Phenols accounted for more than 90% of the metabolites produced by these two cytochromes. The major phenobarbital-inducible cytochrome P-450, Form 2, exhibited practically no catalytic activity (<1% of the other forms) with AAF as a substrate. Since N- and ring-hydroxylation are thought to represent divergent pathways of carcinogen metabolism (activation versus detoxification), the differential occurrence of the various cytochrome forms should affect the balance between these two reaction pathways. In this sense, cytochrome P-450 induction by 2,3,7,8-tetrachlorodibenzo-p-dioxin differentially affects the magnitude and direction of in vitro microsomal metabolism of AAF as a function of age.

INTRODUCTION

The carcinogen AAF3 requires metabolic activation in order to initiate carcinogenesis. The N-hydroxylation of this compound is considered to be the initial activation step, whereas aromatic hydroxylation appears to provide a detoxification pathway (17, 26, 27). These reactions are catalyzed by the cytochrome P-450 monoxygenases (23), which consist of multiple forms of cytochrome P-450 and associated electron transport components (7).

Evidence derived chiefly from the effects of inducing agents on AAF N-hydroxylation activity has implicated the action of specific forms of the cytochrome in AAF activation. AAF N-hydroxylation is induced by TCDD or MC in several species (22, 24) including rabbit (1, 2). Two forms4 of TCDD-inducible cytochrome P-450, Forms 4 and 6, have been isolated from rabbit liver microsomes (8-11). Form 6 catalyzes benzo[a]pyrene hydroxylation more rapidly than the other 3 forms, and the induction of this cytochrome is associated with increases in aryl hydrocarbon [benzo(a)pyrene] hydroxylase activity in neonatal rabbits (20). On the other hand, the induction of Form 4 is similar to that of AAF N-hydroxylase with regard to age and tissue occurrence (1, 2, 13, 20). Therefore, we tested the ability of Form 4, Form 6, and 2 other highly purified cytochrome forms to catalyze AAF N- and ring-hydroxylation.

MATERIALS AND METHODS

Materials. Cytochrome P-450 Forms 2 (12), 3 (8), 4 (10), and 6 (8, 9) were isolated by published procedures. The cytochrome preparations used here had the following specific contents (nmol/mg): Form 2, 16.0, 16.6, and 16.6; Form 3, 14.5, 18.8, and 18.0; Form 4, 16.8, 18.3, and 16.6; and Form 6, 16.0 and 15.8. Cytochrome P-450 reductase was prepared according to the method of Yasukochi and Masters (28) with minor modifications (12). Reductase preparations used in this study had specific activities of 40, 47, and 42 units/mg, where 1 unit of activity corresponds to the reduction of 1 µmol of cytochrome c per min. The isolation of microsomes and the pretreatment of animals are described elsewhere (8-12, 20). Details concerning the characterization of cytochrome P-450, reductase, and microsomes can be found in the references cited above. Synthetic dilauroyl-L-a-lecithin was obtained from Calbiochem, La Jolla, Calif., and NADPH was purchased from Sigma Chemical Co., St. Louis, Mo. The [9-14C]AAF was obtained from New England Nuclear, Boston, Mass., and purified by thin-layer chromatography [chloroform:methanol, 97:3 (v/v)]. The substrate had a specific activity of 68 dpm/µmol.

Methods. The enzymes were reconstituted as described in previous publications from this laboratory (7-12), using 0.2 nmol cytochrome P-450, 0.7 unit of reductase, and 60 µg dilauroyl-L-a-lecithin. When microsomes were used as an enzyme source, an amount corresponding to 0.5 mg of protein was used. The assay mixture contained 0.2 µmol [14C]AAF added in 10 µl dimethyl sulfoxide, 100 nmol NADPH, and 0.15 µmol KCl in 2 ml 20 mM potassium phosphate buffer (pH 7.4). When microsomes were used, 0.2 µmol NaF was also included. The reaction mixtures were incubated for 20 min at 37° with shaking following the addition of NADPH, and the reaction was terminated by removal to ice. Product formation was linear with time over the duration of this incubation period.

AAF and its metabolites were extracted twice into 5-ml portions of diethyl ether after buffering the reaction mixture to pH 6.5 by the addition of 1 ml of 1 M acetate buffer (pH 6.5). The combined diethyl ether extract was evaporated to dryness.
under a stream of nitrogen, and the residue was dissolved in a small volume of methanol containing authentic standards for all the AAF metabolites examined. The AAF metabolites formed by the catalytic action of the 4 reconstituted, purified forms of cytochrome P-450 were separated and analyzed using high-pressure liquid chromatography and liquid scintillation counting as described by Thorgeirsson and Nelson (25) with some modifications. The modified procedure incorporates a mobile phase containing aqueous acetic acid (0.01 M), isopropyl alcohol and Desferal mesylate (0.01%), a chelating agent. The isopropyl alcohol concentration was increased from 28 to 72% over a period of 20 min to effect a linear gradient elution. In this manner, the formation of 7-, 5-, 3-, and 1-OH-AAF and N-OH-AAF were monitored. The following served as equivalent blank controls: zero incubation time; or omission of NADPH, cytochrome, or reductase.

RESULTS

The rates of metabolite formation are shown in Table 1. In terms of overall metabolism, the 4 forms exhibited the following relative order of activities: Form 4 > Form 6, Form 3 > Form 2. Two of the cytochromes, Forms 3 and 6, catalyzed AAF hydroxylation almost exclusively at carbon 7 (Chart 1), whereas Form 4 hydroxylated predominantly the amide nitrogen. These 2 metabolites, 7-OH-AAF and N-OH-AAF, are the 2 most prominent urinary metabolites formed by the rabbit in vivo (6). Form 4, in contrast to the other forms, catalyzed hydroxylation on several positions of the AAF molecule. As was the case with the other forms, 7-OH-AAF was the principal phenolic metabolite produced. Hydroxylation at carbon atoms 1, 3, and 5 accounted for the remaining 35% of the total phenolic products formed. Form 2 did not metabolize AAF to a significant degree.

With regard to carcinogenesis, 2 aspects of AAF metabolism are likely to be relevant. These are the magnitude of AAF metabolism and the direction of AAF metabolism. The latter refers to the balance between detoxification and activation reactions. Aromatic hydroxylation is considered to be a detoxification pathway (17, 26, 27) and in the rabbit is explicated predominantly by the formation of 7-OH-AAF (6). On the other hand, the N-hydroxylation of AAF which has been clearly implicated in the transformation of AAF to its penultimate carcinogenic form (17, 26, 27) is the activation pathway. Forms 3, 4, and 6 catalyze these divergent pathways of carcinogen metabolism to different extents. This can be conveniently illustrated by representing the metabolism of AAF by a vector which is the sum of a vector component A giving the rate of N-hydroxylation or activation and a vector component D representing the rate of aromatic hydroxylation or detoxification as is shown for Form 4 in Chart 2. The vectors for Forms 3 and 6 (not shown) are coincident with the abscissa since these forms catalyze only the detoxification pathway. Metabolic vectors for AAF metabolism catalyzed by cytochromes 3, 4, and 6 display both the magnitude and the direction of AAF metabolism catalyzed by the different cytochrome forms.

The relationship between the microsomal metabolism of AAF and the activities of the purified cytochromes was examined using microsomal preparations from PB-, TCDD-, and non-treated, newborn and adult rabbit livers. The relative occurrences of the 4 cytochromes are different in these preparations (7, 8, 20, 21). The results of these experiments are shown in Table 2. As was seen in the reconstitution experiments, 7-OH-AAF and N-OH-AAF represent the predominant metabolites. Hydroxylation of carbon atoms 1 and 3 did not occur to a significant degree.

Microsomal metabolism can also be represented as a vector (Chart 3). In this case, microsomal metabolism will reflect the vector sum of the catalytic contributions of all the individual cytochromes. The direction of the metabolic vector for purified Form 4 is indicated in Chart 3 for comparison. In this representation, the correspondence between neonate microsomes and the detoxification cytochromes (Forms 3 and 6) is clearly shown by the coincidence of these vectors with the abscissa. The magnitude of neonatal metabolism is lower than the adult activity, reflecting the lower amounts (20%) of cytochrome P-450 in newborn rabbit liver microsomes. Pretreatment of the rabbit liver microsomes from pregnant rabbits with TCDD resulted in a significant increase in the amount of 7-OH-AAF formed. This increased "sensitivity" of the wild type AAF metabolism to TCDD provides an alternative mechanism for TCDD's carcinogenic activity. It is likely that TCDD causes an increase in the magnitude of AAF metabolism with the activation pathway predominating.

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Table 1: Metabolism of AAF by reconstituted forms of cytochrome P-450

<table>
<thead>
<tr>
<th>Metabolite formation</th>
<th>Form 3</th>
<th>Form 4</th>
<th>Form 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-OH-AAF</td>
<td>0.94 ± 0.07</td>
<td>0.58 ± 0.13</td>
<td>1.13 ± 0.15</td>
</tr>
<tr>
<td>5-OH-AAF</td>
<td>0</td>
<td>0.13 ± 0.03</td>
<td>0</td>
</tr>
<tr>
<td>3-OH-AAF</td>
<td>0</td>
<td>0.04 ± 0.01</td>
<td>0</td>
</tr>
<tr>
<td>1-OH-AAF</td>
<td>0</td>
<td>0.14 ± 0.02</td>
<td>0</td>
</tr>
<tr>
<td>N-OH-AAF</td>
<td>0</td>
<td>2.09 ± 0.16</td>
<td>0</td>
</tr>
</tbody>
</table>

*Mean ± S.D. of experiments performed in triplicate with 2 different preparations of each cytochrome.

**Zero, value less than 0.020 mol/min/mol.

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5 C. L. Smith and S. S. Thorgeirsson, manuscript submitted for publication.

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Chart 1: Schematic representation of the AAF molecule. Carbon atoms are numbered to correspond with sites of aromatic hydroxylation.

Chart 2: Vectorial representation of AAF metabolism by rabbit microsomal cytochrome P-450. Form 4. The metabolism of AAF is presented in vector form as described in "Results." The vector shown (>) is the resultant of 2 vectors, D and A, representing the rate of N-hydroxylation and aromatic hydroxylation, respectively. Vectors for Forms 3 and 6 (not shown) are coincident with the abscissa. The units along each axis are mol of product formed per min per mol cytochrome P-450.
Effect of age and inducing agents on the in vitro microsomal metabolism of AAF

The results of 2 experiments are shown and the assay procedures are described in "Materials and Methods." Table 2 and Chart 3. PB treatment does not alter the direction of AAF metabolism in the neonate, although the rate of AAF metabolism is increased relative to untreated neonatal microsomes. However, PB effects a change in both the direction and magnitude of AAF metabolism catalyzed by adult rabbit liver microsomes. Since PB affects the directions of adult and neonatal metabolism differently, even though Form 2 is induced in each case, we suggest that the effect of PB on AAF metabolism is mediated primarily by factors other than the induction of Form 2.

<table>
<thead>
<tr>
<th>Sources of microsomes</th>
<th>7-OH</th>
<th>5-OH</th>
<th>N-OH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control neonate</td>
<td>0.28 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Trace&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Trace&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>TCDD neonate</td>
<td>0.16 ± 0.01</td>
<td>Trace</td>
<td>Trace</td>
</tr>
<tr>
<td>PB neonate</td>
<td>0.80 ± 0.03</td>
<td>0.04 ± 0.01</td>
<td>Trace</td>
</tr>
<tr>
<td>Control adult</td>
<td>0.62 ± 0.03</td>
<td>0.03 ± 0.01</td>
<td>Trace</td>
</tr>
<tr>
<td>TCDD adult</td>
<td>0.50 ± 0.01</td>
<td>Trace</td>
<td>Trace</td>
</tr>
<tr>
<td>PB adult</td>
<td>0.86 ± 0.04</td>
<td>0.12 ± 0.01</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>1.65 ± 0.09</td>
<td>0.10 ± 0.01</td>
<td>0.19 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>2.10 ± 0.05</td>
<td>Trace</td>
<td>1.27 ± 0.03</td>
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<td></td>
<td>2.82 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.31 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>2.41 ± 0.06</td>
<td>0.17 ± 0.01</td>
<td>0.85 ± 0.03</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± S.D. of duplicate determinations.
<sup>b</sup> Trace, values not significantly greater than blank values (p > 0.01).

Chart 3. Vectorial representation of microsomal AAF metabolism. Metabolic vectors are shown for reactions catalyzed by liver microsomes from TCDD-treated (T), PB-treated (P), and control (C), adult (A), and neonate (N) rabbits. -- -- -- -- direction of the vector corresponding to purified cytochrome P-450 Form 4. Induction of cytochrome P-450 by TCDD in newborn rabbits increases the magnitude but not the direction of AAF metabolism. In contrast, both the magnitude and the direction of AAF metabolism are affected in the adult. The units along each axis are nmol of product formed per min per mg of protein.

A metabolic activation hypothesis is currently proposed to explain the initiation of carcinogenesis by AAF (17, 26, 27). As shown in the present study, rabbit cytochrome P-450 Form 4 catalyzes the initial step in the activation of AAF most rapidly, and the induction of this cytochrome P-450 by either TCDD or MC results in an increase in the rate of in vitro AAF N-hydroxylation by adult rabbit liver microsomes. The effect of MC on AAF tumorigenesis has not been determined in the rabbit. MC does affect AAF tumorigenesis in 2 other species, namely, the rat and hamster, but it exhibits disparate effects. Although MC increases in vitro rates of microsomal N-hydroxylation in both species, it decreases AAF carcinogenicity in the rat (18, 19) and increases it in the hamster (3). Thus, AAF carcinogenicity may not be sensitive to increases in the rate of the activation reaction alone.

Since the time scale of carcinogenesis (months to years) is much longer than the time scale of metabolism (min to hr), the final events in carcinogenesis, e.g., the appearance of a neoplasm, might depend less on the rate at which a carcinogen passes through the activation process and to a greater degree on the proportion of the dose which is activated, i.e., the reaction end point. When the amount of a carcinogen (substrate) is limited, divergent pathways may compete for the substrate until it is exhausted, and the end point of the reaction will reflect the balance between the divergent pathways. A kinetic model of this nature has been described by Gillette (4, 5) for the activation of xenobiotics to covalently bound metabolites.

The paradoxical effect of MC on AAF carcinogenesis in the rat and hamster may be explained in this manner. For although MC increases the in vitro rate of N-hydroxylation in both species, it shifts the balance between in vitro aromatic and N-hydroxylation differentially in each species; i.e., MC treatment causes N-hydroxylation to increase relative to ring-hydroxylation in the hamster and decrease in the rat (14, 15). This is reflected by changes in the in vivo urinary excretion of N-OH-AAF. This product, as a percentage of the total administered dose, increases in the hamster but decreases in the rat (14, 15). In this example, the effect of the inducing agent on the direction of AAF metabolism may be a more important determinant of AAF carcinogenesis than is its effect on the magnitude of the activation rate.

The present work indicates that individual cytochromes differ in both the magnitude and direction of the carcinogen metabolism they catalyze. In the case of the 4 cytochromes described...
here, Forms 3 and 6 catalyze almost exclusively the detoxification pathway, whereas Form 4 catalyzes predominantly the activation of AAF. Our results indicate that the major PB-inducible cytochrome, Form 2, does not participate to a significant degree in the metabolism of AAF. Since the occurrence of each cytochrome is dependent on many factors and the relative role of each cytochrome must be integrated with other processes occurring during metabolism and carcinogenesis, it is difficult to predict the impact of these metabolic differences on carcinogenesis. In spite of these uncertainties, carcinogen metabolism plays an often implicit role in the detection of carcinogens by either lifetime animal or short-term in vitro tests, and the outcome of this testing has major health, social, and economic consequences. Thus, there exists a need for a more clearly defined relationship between the elements of carcinogen metabolism and carcinogenicity.

REFERENCES


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