Purified Form of Cytochrome P-450 from Rainbow Trout with High Activity toward Conversion of Aflatoxin B$_1$ to Aflatoxin B$_1$-2,3-epoxide$^1$

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ABSTRACT

Aflatoxin B$_1$, the most potent hepatic chemical carcinogen known, is activated to the putative product aflatoxin B$_1$-2,3-epoxide via a cytochrome P-450-dependent reaction. Mt. Shasta rainbow trout is the most sensitive species known to the hepatocarcinogenic effects of aflatoxin B$_1$. We have previously isolated and purified a minor form of cytochrome P-450 from this strain of rainbow trout, with a $\lambda_{max}$ in the carbon monoxide-reduced difference spectrum of 449.5 nm and a molecular weight of 54,000. In this study, we have compared in a reconstituted system this trout P-450 to trout cytochrome P-448 and rat cytochrome P-450 and P-448 for metabolism and activation of aflatoxin B$_1$. Trout cytochrome P-450 had much higher activity towards aflatoxin and a greater degree of regioselectivity in the formation of aflatoxin B$_1$-3,4-dihydroxy-3,4-dihydrodiol and was much more efficient in producing aflatoxin B$_1$-covalent adducts with DNA. The existence of such a form of cytochrome P-450 in Mt. Shasta rainbow trout may be responsible for the acute sensitivity of this strain to the carcinogenic effects of aflatoxin B$_1$.

INTRODUCTION

Aflatoxin B$_1$, an extremely potent hepatocarcinogen, is one of the few chemicals for which there is a strong correlation between dietary intake levels and cancer incidence in human populations (30, 43). AFB$_1$ is metabolized by the P-450-dependent mixed-function oxidase system to a variety of more polar metabolites, including AFM$_1$, AFQ$_1$, and the putative AFB$_1$-2,3-epoxide (11, 12). The formation of M$_1$ and AFQ$_1$ represents detoxication, while AFB$_1$-2,3-epoxide is an activated metabolite which binds covalently to protein, RNA, and DNA (5, 8, 13, 23). Studies have shown that the degree of AFB$_1$-metabolite-DNA adduct formation agrees well with the carcinogenic potency and acute toxicity of AFB$_1$ in various tissues and species (5, 23, 27, 38). Furthermore, hydrolysis and isolation of the DNA adducts have shown that 90% of the AFB$_1$ metabolites bound can be rationalized as being derived from AFB$_1$-2,3-epoxide (7).

As is the case for other carcinogens [such as benzo(a)pyrene], the existence of multiple mixed-function oxidase pathways leading to deactivated and activated products is due to multiple forms of P-450 with distinct regioselectivity toward the substrate. AFB$_1$, hydroxylation at position 4 to form AFM$_1$, is catalyzed, preferentially, by BNF-induced P-448 (11, 12, 24, 45), whereas formation of AFQ$_1$ and AFB$_1$-2,3-epoxide (or diol) is mediated by one or more forms of PB-induced P-450 (11, 12, 45). Mt. Shasta rainbow trout is the most sensitive species known to AFB$_1$, and it is currently being used as an animal model for AFB$_1$-induced hepatocarcinogenesis (34). Rainbow trout, as all species of fish tested to date, are not affected by "PB-type" inducers but are quite responsive to (P-448) induction by compounds such as BNF (18). Using techniques developed in our laboratory (39), we have purified multiple forms of P-450 from BNF-treated rainbow trout of this strain. Comparison of the main purified trout form with rat P-450 and rat P-448 suggests that it is more like rat P-448 with regard to spectral properties and substrate specificity (40). More recently, a second purification of trout P-450 was undertaken (41, 42), and the main form isolated was a P-448 as found previously. In addition, the second purification process also yielded a P-450 type of cytochrome. This minor form had a molecular weight lower than the major P-448 and a $\lambda_{max}$ in the CO-reduced difference spectrum of 449 to 450 nm. In the present study, we compared the AFB$_1$ metabolite profile and total DNA binding catalyzed by purified rat and trout P-450 and P-448 in a reconstituted system containing purified rat NADPH-P-450 reductase and lipid.

MATERIALS AND METHODS

Chemicals. [G-14C]AFB$_1$ (50 mCi/mmol) and [G-3H]AFB$_1$ (14 Ci/mmol) were purchased from Moravek Biochemicals (Brea, Calif.), diluted to a known specific activity with unlabeled AFB$_1$ from Sigma Chemical Co. (St. Louis, Mo.), and purified by thin-layer chromatography. AFM$_1$, AFQ$_1$, calf thymus DNA, dilauroyl-L-phosphatidylcholine and deoxycholate were also from Sigma. Nitrocellulose membranes (0.45 $\mu$m; 25 mm in diameter) were from Schleicher and Schuell (Keene, N. H.). AFB$_1$-2,3-diol was supplied by Dr. Gerald N. Wogan of Massachusetts Institute of Technology (Cambridge, Mass.). [7-3H]Styrene oxide (88 mCi/mmol) was obtained from Amersham/Searle Corp. (Arlington Heights, Ill.). Purification of Enzymes. P-450 and P-448 were purified from rats pretreated with PB and BNF, respectively (9). Both rat P-450 and P-448 were homogenous on SDS-PAGE (17) and had specific contents of 13.8

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3 Abbreviations used are: AFB$_1$, aflatoxin B$_1$; AFM$_1$, aflatoxin M$_1$; AFB$_1$-epoxide; BNF, 3-naphthoflavone; PB, phenobarbital; rat P-450, the major rat liver microsomal cytochrome P-450 induced by phenobarbital; rat P-448, the major rat liver microsomal cytochrome P-450 induced by 3-naphthoflavone; trout P-450, a minor liver microsomal cytochrome P-450 from 3-naphthoflavone-induced rainbow trout (also called trout LM$_1$); trout P-448, the major liver microsomal cytochrome P-450 from 3-naphthoflavone-induced rainbow trout (also called LM$_2$); AFB$_1$-2,3-diol, aflatoxin B$_1$-2,3-dihydroxy-2,3-dihydrodiol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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and 11.6 nmol/mg, respectively. NADPH-P-450 reductase, purified from rats pretreated with polychlorinated biphenyls (9), was homogenous on SDS-PAGE and had a specific activity of 32 \( \mu \text{mol/min/mg} \). Trout P-450 and P-448 were purified from BNF-fed rainbow trout (39) to specific contents of 10.8 and 11.9 nmol P-450 per mg, respectively, were highly purified as determined by SDS-PAGE, and were easily resolved using this SDS-PAGE system (45), with apparent minimum molecular weights of 58,000 (P-448) and 54,000 (P-450). Hepatic microsomes from untreated and BNF-treated trout were prepared as described previously (39).

Reconstitution and Metabolic Profiles. The reconstituted system was identical to that of Yoshizawa et al. (45), except that NADPH was used in place of a NADPH-generating system, and \([\text{G}-1^4\text{C}]\)AFBi was used as the substrate. Reactions were initiated with NADPH and allowed to proceed for 20 to 30 min at either 30° (trout) or 37° (rat) in the dark with shaking. The reactions were terminated by the addition of 2.5 ml of ice-cold methanol. Known amounts of unlabeled AFM, AFQ, and AFB-2,3-diol were added to determine the recovery efficiency (80%) and to identify by coelution metabolites on high-pressure liquid chromatography (Spectra Physics Model 8000). After centrifugation, the mixtures were evaporated under reduced pressure (35°), and the residues were taken up in methanol, transferred to small vials, and evaporated under \( \text{N}_2 \). The residues were redissolved in 100 \( \mu \)l of 10% acetonitrile in water and injected directly onto a Zorbax octadecylsilane (4.5 mm x 25 cm) column. Resolution of AFB metabolites was achieved by a slight modification (using a linear gradient of 0 to 40% acetonitrile) of the method of Neal and Colley (4, 25). The elution of AFB and metabolite standards was determined by monitoring the absorbance at 365 nm (Spectra Physics Model 770), and the AFB, metabolites were quantitated by collecting 0.5-min fractions directly into scintillation vials and counting (Packard Tri-Carb Model 3375).

Binding of AFB, Metabolites to DNA. For analysis of DNA binding, the reconstitution system was altered as described by Yoshizawa et al. (45) by the addition of 150 \( \mu \)g of calf thymus DNA and with \([\text{G}-3^\text{H}]\)AFBi as substrate. The procedure for phenol extraction and binding to filters was modified slightly in that a solution of phenol:cresol:8-hydroxyquinoline (100:14:0.1) saturated with 0.1 \( \text{M} \) Tris (pH 8.0) was used in place of water-saturated phenol for protein extraction, and nitrocellulose filters, rather than glass fiber filters, were used to bind DNA.

Other Assays. P-450 (29) and NADPH-cytochrome c reductase (15) were assayed by standard spectrophotometric techniques. Protein was determined by the method of Lowry et al. (22) with bovine serum albumin as standard. SDS-PAGE was performed using a slight modification (39) of the Laemmli method (17).

RESULTS

Metabolic profiles of AFB, catalyzed by purified rat or trout P-450 reconstituted with rat NADPH-P-450 reductase, were analyzed by high-pressure liquid chromatography, and a total of 5 peaks were resolved. In addition to the 3 standards, AFB-2,3-diol, AFQ, and AFM, 2 peaks of unknown identity (not seen in blanks) eluted earlier than did the diol (Chart 1). These unidentified peaks were termed water fraction and Peak 1, and the absolute amounts were quantitated along with the radioactivity coeluting with the 3 unlabeled standards (Table 1). Water fraction accounted for 6 to 10% of the total metabolites in all samples, while Peak 1 was the major metabolite for rat P-450 and trout P-448.

Rat P-450 and P-448 formed AFQ, and AFM, respectively, as major metabolites (Table 1), consistent with previous reports with PB or BNF microsomes and reconstituted P-450 and P-448 (11, 12, 45). The PB-induced rat P-450 was also more effective in formation of AFB-2,3-diol than was rat P-448, which is also consistent with previous findings with microsomes (11, 12). When trout P-450 and P-448 were examined in a reconstituted system, it was found that the trout P-450 had a much higher turnover with AFB, than did trout P-448 or either rat enzyme (Table 1). Furthermore, trout P-450 exhibited a high regioselectivity toward formation of the AFB-2,3-diol. Formation of this activated metabolite was over 13-fold greater with trout P-450 compared to rat P-450 and accounted for 72% of total AFB, metabolized by trout P-450 (17% for rat P-450). Trout P-448 had much lower overall activity towards AFB, than did trout P-450, and AFB-2,3-diol accounted for less than 6% of the total metabolites.

The relative amount of AFB-2,3-diol correlated with the degree of total AFB, binding to DNA (Table 1). Trout P-450 produced 22-fold more DNA binding than did rat P-450 and over 130-fold more than rat P-448. The amount of AFB, DNA binding catalyzed by trout P-448 was actually less than background (no NADPH) incubations.

In order to compare the results for purified trout P-450 and P-448 with microsomes from BNF-treated (from which they were purified) and untreated trout, AFB, metabolite profiles and DNA binding were examined using unsolubilized microsomes. Microsomes from untreated trout yielded profiles very similar to those of purified trout P-450 (Table 2). Pretreatment with BNF produced microsomes that formed considerably less AFB,2,3-diol and much more AFM. The relative amount of total AFB, bound to DNA, again, correlated with the degree of AFB,2,3-diol produced.
The potency of AFB₁ as a hepatocarcinogen varies between species and is regulated by diet, sex, hormones, and pretreatment with PB or BNF (2, 3, 12, 13, 24, 35). Many studies have demonstrated that AFB₁ is metabolized and activated to its ultimate carcinogenic form, the putative AFB₁-2,3-epoxide, by P-450 (11, 12, 19, 24, 25). The relative risk of a particular animal on April 16, 2017. © 1983 American Association for Cancer Research. cancerres.aacrjournals.org Downloaded from to DNA (5, 23, 38). Formation of this toxic metabolite in mammals is due primarily to the action of PB-inducible P-450(s). Other metabolites include AFQ, and AFM₁. The former is also formed most efficiently by PB-induced P-450 (45), whereas production of AFM₁ is a BNF-inducible, P-448, reaction (45), the activity of which cosegregates with the Ah locus (11, 12, 32).

Reconstituted trout P-450 was at least an order of magnitude more effective in formation of AFB₁-2,3-diol and covalent AFB₁-DNA adducts through the putative AFB₁-2,3-epoxide than was rat P-450 or P-448, whereas trout P-448 was much less effective.

Studies here with intact trout microsomes showed that the major metabolite from untreated trout is the AFB₁-2,3-diol. Pretreatment of trout with BNF reduced total AFB₁-2,3-diol formation with microsomes, while increasing the amount of AFM₁ formed. These results are consistent with the protective effect of BNF pretreatment towards AFB₁-induced hepatocarcinogenesis in rainbow trout (14) but in conflict with the previous finding that the main metabolite formed in vitro by postmitochondrial supernatant from trout is aflatoxicol (21). Aflatoxicol formation is a cytosolic process, which explains the lack of its formation in the present study. It has been postulated that formation of aflatoxicol and subsequent reversion to AFB₁ may determine the sensitivity of an animal to AFB₁ (33), presumably by acting as a storage reservoir for AFB₁, thus prolonging its action. The equilibrium between aflatoxicol and AFB₁ may have some effect, but since AFB₁-2,3-epoxide is the ultimate carcinogen in any case, the relative amount of the highly active trout P-450 form reported here is probably of greater importance.

It would be interesting to examine hepatic nuclear membrane from trout for the presence of this P-450 with high activity towards AFB₁, since activation of AFB₁ close to the critical target may be of greater significance than the occurrence of this isozyme in the endoplasmic reticulum. Activation of AFB₁ by rat nuclear P-450 has been shown to occur in the same manner as that in rat microsomes (37). The levels of various isozymes can be assayed with antibodies and the recently developed immunochemical assays for quantitating the relative amounts of specific P-450 isozymes (10, 31). Preparation of rabbit antibodies to trout P-450 and P-448 and such immunoanalyses are currently under way in our laboratory.

The lack of AFB₁-2,3-diol formation by trout found in previous studies (21, 33) could be due to at least 2 factors. (a) Incubations should be carried out in Tris Buffer, which allows for the isolation of a AFB₁-2,3-diol-Tris complex. If other buffers are used, the AFB₁-2,3-diol is lost due to very rapid covalent binding to tissue macromolecules (26). (b) The AFB₁-2,3-diol-Tris complex is probably not readily extractable into organic solvents, leading to an underestimation of its formation.

The results with intact microsomes, along with the observed ratio of 1:5 (41) of purified P-450:P-448 from BNF-treated trout microsomes, suggests that this P-450 may be a constitutive form, the relative amount of which could be reduced following BNF treatment. Studies in mammals have shown that BNF pretreatment not only markedly increases P-448 but concurrently depresses the level of other, presumably constitutive, forms (6, 31).

It was somewhat surprising, given the relatively large increase in formation of AFM₁ with microsomes from BNF-treated trout (Table 2), that purified trout P-448 was not active in production of AFM₁ in a reconstituted system (Table 1). At least 3 possibilities could be proposed for such a result. (a) The first involves a selective loss, during the purification, of a BNF-induced P-450 isozyme with high regioselectivity toward formation of AFM₁. (b) Trout P-448 may have been altered somehow during purification or inhibited by residual detergent, such that reconstituted cata-

### Table 1

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Water fraction</th>
<th>AFB₁-2,3-diol</th>
<th>AFQ₁</th>
<th>AFM₁</th>
<th>Total</th>
<th>AFB₁ bound to DNA</th>
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<td>Trout P-450</td>
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<td>404</td>
<td>39</td>
<td>27</td>
<td>561</td>
<td>131</td>
</tr>
<tr>
<td>Trout P-448</td>
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<td>5</td>
<td>14</td>
<td>12</td>
<td>86</td>
<td>ND*</td>
</tr>
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</table>

* ND, not detectable.

### Table 2

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<th>Microsomes</th>
<th>Water fraction</th>
<th>AFB₁-2,3-diol</th>
<th>AFQ₁</th>
<th>AFM₁</th>
<th>Total</th>
<th>AFB₁ bound to DNA</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>139</td>
<td>484</td>
<td>38</td>
<td>32</td>
<td>847</td>
<td>36</td>
</tr>
<tr>
<td>BNF-treated</td>
<td>66</td>
<td>88</td>
<td>33</td>
<td>160</td>
<td>431</td>
<td>9</td>
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</tbody>
</table>
lytic activity of the purified isozyme towards formation of AFM, was much lower than when P-448 was present in unsolubilized microsomes. (c) A more probable explanation may be that the conditions used for reconstitution were not optimal for trout P-448-catalyzed formation of AFM. The conditions used were those used in a study on purified rat P-450 and P-448 (45), with the exception that the temperature was lowered from 37°C to 30°C for the trout isozymes. No attempt was made to optimize conditions such as temperature, pH, amount and type of lipid, or the concentration of trout P-448 or rat NADPH-P-450 reductase. Cytochrome b5, which has been shown to be stimulatory (16, 28) or obligatory (36) for some reconstituted P-450 reactions, was not added to incubations containing trout P-448. However, when b5 was added to reconstituted rat or trout P-450, the results were equivocal, with some metabolites increased and others decreased (data not shown).

The inability of trout P-448 to catalyze formation of AFM does not detract from the significance of our finding that trout P-450 exhibits a high turnover toward AFB1 and is regioselective for conditions used for reconstitution were not optimal for trout P-450-catalyzed formation of AFM. The conditions used were those used in a study on purified rat P-450 and P-448 (45), with the exception that the temperature was lowered from 37°C to 30°C for the trout isozymes. No attempt was made to optimize conditions such as temperature, pH, amount and type of lipid, or the concentration of trout P-448 or rat NADPH-P-450 reductase. Cytochrome b5, which has been shown to be stimulatory (16, 28) or obligatory (36) for some reconstituted P-450 reactions, was not added to incubations containing trout P-448. However, when b5 was added to reconstituted rat or trout P-450, the results were equivocal, with some metabolites increased and others decreased (data not shown).

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