Characterization of Benzo(a)pyrene Hydroxylase of Trout Liver

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SUMMARY

Trout liver microsomes contained as much as 0.40 nmole of cytochrome P-450 per mg of protein and a NADPH-pyruvate c reductase activity of 23 nmole of cytochrome c reduced per mg of protein per min at 22°. Associated with these was a high benzo(a)pyrene hydroxylase activity, which required NADPH and O2 and was inhibited by CO. With thin-layer chromatography, at least five metabolites could be identified (including dihydrodiols, phenols, and quinones of benzo(a)pyrene).

Inhibitors such as 2-diethylaminoethyl-2,2-diphenylvaler-ate, anilopyrine, metyrapone, pyridine, n-octylamine, and 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane were relatively ineffective in inhibiting trout benzo(a)pyrene hydroxylase. Typical inhibitors of 3-methylcholanthrene-induced cytochrome (P-448), such as α-naphthoflavone, zoxazolamine, and testosterone, were effective, however.

With benzo(a)pyrene it was possible to induce type I spectral change in trout cytochrome P-450. In spite of the many enzymatic characteristics of cytochrome P-448, trout cytochrome P-450 had maximum absorbance at 450.6 nm. When in reduced form and complexed with CO, the ethyl isocyanide gave an interaction spectrum with reduced trout liver cytochrome P-450 resembling that of control rat.

INTRODUCTION

In the endoplasmic reticulum of mammalian liver cells, there is an enzyme system of utmost importance in the detoxication and activation of xenobiotics. This cytochrome P-450-linked MFO3 system is believed to be responsible for the activation of numerous compounds, including several carcinogenic ones (15). Until recently, it was widely held that the MFO system is absent at the level of fish in the evolutionary tree (11). However, several species of fish have now been shown to possess microsomal cytochrome P-450 (8, 28) and MFO activity (9, 10). It has also been established that trout, in particular, are very sensitive to some chemical carcinogens and develop hepatomas readily (18, 27). This suggests the presence of a carcinogen-activating system in trout liver. In this paper we describe in some detail a highly active AHH of trout liver microsomes [a preliminary communication of some aspects of this study appeared earlier (2)].

MATERIALS AND METHODS

Animals. The fish used in our experiments were hatchery-reared Salmo trutta lacustris, a local lake trout (Hatchery of Oulujoki Pty. Ltd., Montta, Finland). The trout were sexually immature (about 1.5 years old), and they were kept at least 1 week in our aquarium at 4–6° before use. The water of the aquarium was filtered through charcoal and was changed every other day. Both in the hatchery and in our aquarium, the fish were fed Ewos Salmon Grower F159 fodder (Astra-Ewos AB, Södertälje, Sweden) 10 g/kg of fish per day. The rats used in our experiments were adult males of the Sprague-Dawley strain, weighing about 250 to 300 g. They were fed with a commercial pellet diet (HankkiCo- operative, Finland), except for 1 group of rats that received Ewos Salmon Grower F159 fodder from weaning to maturity.

Pretreatment of Animals. The trout received no pretreatment. Unless otherwise specified, the rats received a pretreatment of phenobarbitone [0.5% (w/v) in drinking water, ad libitum, for 1 week; discontinued 24 hr prior to killing]; and 3-MC [25 mg/kg in vegetable oil administered i.p. on 3 consecutive days (last dose 24 hr prior to killing)]. The animals were killed by decapitation; the livers were removed immediately thereafter and homogenized in 4 volumes of 0.1 M potassium phosphate-sodium phosphate buffer, pH 7.4, with a Teflon-glass Potter-Elvehjem homogenizer. The homogenates were centrifuged at 1,000 x g for 5 min (at times this centrifugation was omitted), and the supernatant obtained was centrifuged at 10,000 x g for 20 min. The 10,000 x g supernatant was centrifuged at 100,000 x g for 60 min, and the pellet obtained (microsomes) was resuspended in 0.1 M potassium phosphate-sodium phosphate buffer so that 1 ml contained microsomes from 1 g of liver. The centrifugations were carried out with refrigerated MSE-25 high-speed and MSE-50 ultracentrifuges. All other procedures were carried out at 0–4°.

Microsomes used for the recording of cytochrome P-450 spectra and for the ethyl isocyanide spectral studies were prepared as follows. Livers were homogenized in 4 volumes (w/v) of 0.25 M sucrose with a Teflon-glass Potter-Elvehjem homogenizer. The homogenate were centrifuged at 10,000 x g for 15 min, and the supernatant obtained was centrifuged at 105,000 x g for 60 min. The microsomal pellet was resuspended in the starting volume of 0.15 M KCl, and this suspension was centrifuged at 105,000 x g for 60 min. The microsomes obtained were then suspended in 0.1 M potassium phosphate-sodium phosphate buffer at pH values be-
low 7.4 and in 0.1 M Tris-HCl at pH values above 7.4.

**Assay Methods.** To determine the amount of cytochrome P-450 present, we suspended the microsomal pellets in 0.1 M potassium phosphate-sodium phosphate buffer (pH 7.4) to obtain a suspension containing microsomes from 100 mg of liver (wet weight) per ml of suspension. The suspension was then bubbled with carbon monoxide for 30 sec and was introduced into 2 quartz spectrophotometer cells. The contents of the experimental cells were reduced with sodium dithionite, and their visible spectra were recorded against the unreduced reference sample. An absorbance of 450 to 490 nm was measured. This is essentially the method described by Greim et al. (13). The method of Omura and Sato (23) was also used, and we assumed that the extinction coefficient determined by them was applicable also to the trout cytochrome P-450.

The ligand interaction spectra were recorded as the difference between the spectra of cytochrome P-450-ligand and cytochrome P-450 without a ligand. In the case of BP, it was necessary to use a tandem cell arrangement to deduct the absorbance due to BP alone.

The NADPH-cytochrome c reductase activity was determined by the method of Masters et al. (21) at 22°. Protein was measured by the biuret method (19), with bovine serum albumin as standard.

The fluorometric assay of the AHH activity was carried out according to the method of Nebert and Gelboin (22). The metabolites of BP produced in *in vitro* incubations were detected and quantitated according to the methods of Sims (25) and of Borgen et al. (6). One hundred nmoles of tritiated BP (about 800,000 cpm) were incubated in the presence of a cofactor mixture (KCl, 50 mM; MgCl₂, 2.5 mM; glucose 6-phosphate, 1.5 mM; NADP, 62.5 μM; glucose-6-phosphate dehydrogenase, 5 units) and the trout liver microsomal suspension (250 μl, corresponding to about 4 mg of protein) in a total volume of 4 ml. Normally, the incubations with rat liver microsomes were carried out at 37°, and those with trout liver microsomes were carried out at 30° [the “optimum” temperature for trout liver MFO is approximately 29° (3)]. After a 15-min incubation, the mixture was extracted twice with 6 ml of ethyl acetate, and the extracts were combined and dried with Na₂SO₄. The ethyl acetate was evaporated under a vacuum, and the residue was meditated with Na₂SO₄. The ethyl acetate extracts were separated by thin-layer chromatography for UV fluorescence bands, which were marked, and cut off. Fractions were put into vials for scintillation counting, with Insta-Gel as counting medium.

For identification, the extract of metabolites formed during the incubation of [G-3H]BP with trout liver microsomes and the cofactor mixture was chromatographed in suitable concentrations with nonradioactive standard compounds as carriers. The fluorescent spots due to the standards with trout liver microsomes were carried out at 37°, and the corresponding soluble fraction (containing material from 20 mg of liver per ml) was used for scintillation counting.

**Chemicals.** Glucose 6-phosphate, glucose-6-phosphate dehydrogenase, NADP, α-naphthoflavone, n-octylamine, and testosterone were obtained from Sigma Chemical Co., St. Louis, Mo.; metyrapone was from Ciba Pharmaceutical Co., Summit, N. J.; and zoxazolamine was from McNeil Laboratories, Inc., Fort Washington, Pa. SKF 525A was a generous gift from Smith, Kline and French Laboratories Ltd., Welwyn Garden City, England. The BP metabolites used as reference compounds were kindly donated by H. V. Gelboin, NIH, Bethesda, Md., and P. Sims, Chester Beatty Research Institute, Institute of Cancer Research, London, England. [G-3H]BP was obtained from The Radiochemical Centre, Amersham, England. All other chemicals were obtained from E. Merck, Darmstadt, West Germany.

**RESULTS**

The hepatic cytochrome P-450 levels of trout were relatively high, as much as 10 nmoles/g of liver (wet weight) in the homogenate (Chart 1; Table 2) or 0.04 n mole/mg of protein in the microsomes (Chart 2; Table 2). The NADPH-cytochrome c reductase activity was determined by Masters et al. (21) at 22°. Protein was measured by the biuret method (19), with bovine serum albumin as standard.

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For identification, the extract of metabolites formed during the incubation of [G-3H]BP with trout liver microsomes and the cofactor mixture was chromatographed in suitable concentrations with nonradioactive standard compounds as carriers. The fluorescent spots due to the standards were removed, and the content of radioactive metabolites was determined by scintillation counting. The nonradioactive standards were 1,6-quinoine, 3,5-quinoine and 6,12-quinoine of BP; 3-hydroxy-BP; 9-hydroxy-BP; 7,8-dihydrodiol and 4,5-dihydrodiol of BP (gift from H. V. Gelboin); and cis-4,5-dihydrodiol of BP (gift from P. Sims). Two to 5 nmoles of each standard were applied per spot on a thin-layer chromatography plate.

A large-scale extraction of the metabolites produced by trout liver microsomes was also performed, and the metabolites were separated by thin-layer chromatography for UV spectroscopy to confirm the identities of the products (5).

A Shimadzu MPS 50L spectrophotometer was used for all spectral studies, and Amino-Bowman spectrophotometer was used for fluorometric measurements, and a Packard Model 3320 liquid scintillation spectrometer was used for scintillation counting.
was the major metabolite found in trout liver microsomes, (Chart 3). dihydrodiols of BP, respectively) and Metabolites V and VI versus 0.027 in male rats) (1). This activity was clearly asso
ted with the microsomal fraction (Chart 2). The BP hyd
oxylase activity in the homogenate.

The activity found in the subcellular fractions could be accounted for by
the activity found in the homogenate.

Associated with cytochrome P-450 and NADPH-cytochrome c reductase activity into different subcellular fractions was similar to that of cytochrome P-450. In the case of NADPH-cytochrome c reductase, all of the recoverable activity in the subcellular fractions could be accounted for by the activity found in the homogenate.

Using a series of inhibitors, we further characterized the nature of the trout BP hydroxylase. SKF 525A (200 µM) and aminopyrine (10 mM) [these caused reduction in BP hydroxylase activity (V_max = 0.140 nmole/mg of microsomal protein per min versus 0.027 in male rats) (1)] this activity was clearly associated with the microsomal fraction (Chart 2). The BP hydroxylase activity of trout liver microsomes required NADPH and O_2, and was inhibited by CO (Table 1). The BP hydroxylase activities given above were measured by the method of Nebert and Gelboin (22), with the formation of 3-hydroxy-BP as a measure of BP metabolism. Although 3-hydroxy-BP (Metabolite IV in Chart 3) (and perhaps also 9-hydroxy-BP) was the major metabolite found in trout liver microsomes, several others, such as Metabolites I and II (9,10- and 7,8-dihydrodiols of BP, respectively) and Metabolites V and VI (quinones of BP), were formed in significant quantities (Chart 3).

Using a series of inhibitors, we further characterized the nature of the trout BP hydroxylase. SKF 525A (200 µM) and aminopyrine (10 mM) [these caused reduction in BP hydroxylase activity to 40 and 92% of controls, respectively (2)]. metyrapone, pyridine, n-octylamine, and 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane were relatively ineffective inhibitors of trout and 3-MC-pretreated rat BP hydroxylase (Chart 4). On the other hand, α-naphthoflavone was very effective in inhibiting the trout and the 3-MC-pretreated rat liver microsomal AHH, but not that of the control rats. Similar findings were obtained with zoxazolamine (500 µM), where the AHH activities were 18, 51, and 111% of the uninhibited levels for trout, 3-MC-pretreated rats, and control rats, respectively.
Trout liver microsomal cytochrome P-450 gave interaction spectra with a variety of type II ligands (aniline, n-octylamine, cyanide) and the reversed "type I" spectrum with 1-butanol (3). However, none of the well-known type I ligands gave a type I interaction spectrum (4). BP was an exception, in that it caused spectral changes characteristic of the so-called type I spectrum only if the absorbance due to BP itself was overlooked and eliminated to some extent by a special cell arrangement in recording the spectrum (Chart 5). Ethyl isocyanide caused a characteristic spectral change in the cytochrome P-450 spectrum with 2 absorbance maxima (455.7 and 428.7 nm for trout) (Chart 6).

The ratio of absorbances at these 2 wavelengths ($\Delta A_{455.7-428.7}$) increased markedly as a result of 3-MC pretreatment of rats, due to the formation of cytochrome P-448. However, this ratio was lowest for trout (Table 2).

The pH dependence of the ethyl isocyanide-cytochrome P-450 interaction spectrum indicated a clear distinction between the rat cytochrome P-448 and the trout cytochrome P-450 (Chart 7).
The final concentration of ethyl isocyanide was 3.5 mM after the addition of sodium dithionite at pH 7.4. The spectrophotometric cells was 2 mg/ml. The spectrum was recorded in the Cytochrome P-450 microsomal protein of the Cytochrome P-450 enzyme complex, as obtained with trout (A), control rat (B), 3-MC-pretreated rat (C), and phenobarbital-pretreated rat (D) liver microsomal samples. ---, ethyl isocyanide-induced spectral change in the above-mentioned microsomal samples. The spectra were recorded at pH 7.4 2 min after the samples were reduced with sodium dithionite.

Table 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cytochrome P-450</th>
<th>Cytochrome P-450*</th>
<th>Ethyl isocyanide difference spectrum ratio (ΔA_{395-505})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmoles/mg of protein</td>
<td>% of control</td>
<td>nmoles/g of tissue</td>
</tr>
<tr>
<td>Control rat (n = 8)</td>
<td>0.316 ± 0.071*</td>
<td>125</td>
<td>16.44</td>
</tr>
<tr>
<td>Trout (pooled)</td>
<td>0.396 ± 0.047</td>
<td>125</td>
<td>9.96</td>
</tr>
<tr>
<td>Phenobarbital-pretreated rats</td>
<td>0.825 ± 0.158</td>
<td>261</td>
<td>57.56</td>
</tr>
<tr>
<td>rats (n = 8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-MC-pretreated rats (n = 5)</td>
<td>0.424 ± 0.065</td>
<td>134</td>
<td>19.92</td>
</tr>
<tr>
<td>3-MC-pretreated rats (n = 5)</td>
<td>0.466 ± 0.045</td>
<td>148</td>
<td>21.44</td>
</tr>
</tbody>
</table>

* Duplicate determinations on pooled homogenates of each group.

* Mean ± S.D.

* 3-MC pretreatment, 1 dose of 5 mg of 3-MC per kg i.p.

* 3-MC pretreatment, 3 doses of 25 mg to 3-MC per kg i.p.
Effect of Ewos Salmon Grower F159 trout fodder on male rat hepatic cytochrome P-450 and BP hydroxylase activity

From weaning to maturity the experimental group was fed the above diet exclusively. Microsomes were prepared and determinations were made as described above. None of the differences between 2 groups was statistically significant. There was no statistically significant difference in the microsomal protein content of the 2 groups.

BP hydroxylase activity in presence of inhibitors (% of control)

<table>
<thead>
<tr>
<th>Rats</th>
<th>BP hydroxylase (FU/g of liver/min)</th>
<th>SKF 525A (200 µM)</th>
<th>Aminopyr-</th>
<th>α-Na-</th>
<th>Absorbance peak of reduced cytochrome P-450-CO (nm)</th>
<th>ΔA453-450/unit wt of microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control rats (n = 5)</td>
<td>3453 ± 787*</td>
<td>37</td>
<td>39</td>
<td>80</td>
<td>449.9 ± 0.1†</td>
<td>0.50 ± 0.02†</td>
</tr>
<tr>
<td>Rats fed with Trout Fodder F159 (n = 6)</td>
<td>4447 ± 993</td>
<td>40</td>
<td>46</td>
<td>93</td>
<td>450.2 ± 0.1</td>
<td>0.63 ± 0.03</td>
</tr>
</tbody>
</table>

* Mean ± S.D.
† Mean ± S.E.

DISCUSSION

Trout have been found to be susceptible to carcinogenesis (26, 27). Although there is strong evidence that the high incidence of hepatoma in trout is a result of chemical carcinogens, the present hypothesis on chemical carcinogenesis involves in situ activation of a procarcinogen (17). Such activation seems to result from the MFO activity of the endoplasmic reticulum. Trout liver contains a considerable amount of cytochrome P-450 in the microsomal fraction, i.e., in the endoplasmic reticulum. Also, there is a considerable amount of NADPH-cytochrome c reductase activity. Although the postmicrosomal fraction contains some NADPH-cytochrome c reductase activity, this may be an artifact, indicating only that this enzyme is slightly less strongly attached to the trout endoplasmic reticulum than it is in the mammalian system. This enzyme complex very readily metabolizes a well-known carcinogen, BP, requiring O2 and NADPH. Whether carcinogenic intermediates are formed to a harmful extent depends on the subsequent events, which eliminate reactive intermediates (e.g., glutathione conjugation and epoxide hydrase activity), but the primary step in activation of BP occurs readily in trout.6

The BP hydroxylase activity of trout was readily inhibited by CO (Table 1), confirming the monooxygenase nature of the system. However, it was found that SKF 525A is not a very strong inhibitor of BP hydroxylase or other drug oxidations by trout (3). Subsequent screening with a number of known MFO inhibitors has revealed a pattern similar to that found by Goujon et al. (12) (Chart 4). None of the substances known to inhibit strongly the mammalian cytochrome P-450 was effective on the trout cytochrome P-450. However, α-naphthoflavone, zoxazolamine, and testosterone,6 which are known to inhibit mammalian cytochrome P-448, were very effective inhibitors in trout, suggesting that the trout system is a typical cytochrome P-448. However, there is major evidence against this: (a) the Soret peak of the CO-complexed reduced cytochrome was slightly above 450 nm; (b) ethyl isocyanide spectrum gave a ratio of 0.35 for ΔA455-430/ΔA455 at pH 7.4; and (c) the pH of equal absorbance at 455 and 430 nm for trout liver cytochrome P-450 was between those obtained for control and phenobarbital-pretreated rat liver cytochrome P-450 and was considerably higher than that of rat cytochrome P-448. Furthermore, there is no evidence to date suggesting that the trout studied received inducing compounds; the same food failed to cause induction of AHH when given to rats (Table 3). Therefore, it seems possible that we are dealing with an anomalous cytochrome P-450 that may be of con-

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6 Preliminary investigations with Salmonella typhimurium (strains TA 1538 and TA 98) indicate that trout liver postmitochondrial supernatant produces mutagenic metabolites from BP, 2-aminofluorene, and 2-acetylaminofluorene (1).

With mice it has been possible to discriminate between control and 3-MC-pretreated animals with testosterone as an inhibitor (12). In our study testosterone also effectively inhibited the AHH of control rats.
stitative nature. In that case, the function of this enzyme remains quite uncertain. The strong inhibitory effect of testosterone and some preliminary experiments on the metabolism of steroids give some indication that the enzyme is involved in the metabolism of steroids.

However, the fact remains that trout metabolize polycyclic hydrocarbons very readily and that the fish are very susceptible to chemical carcinogens such as aflatoxins (14). In fish there may be considerable species differences; it has been reported that some nonsalmonid aquarium fish are not susceptible to polynuclear aromatic carcinogens although they are very sensitive to other types of carcinogens tested (24).

Although BP has not been shown to produce hepatoma or other cancer in trout experimentally, it is a very suitable substrate for metabolic studies of a potential carcinogen in fish, since the presence of considerable quantities of BP in the aquatic environment is an established fact (7, 16). The metabolites of BP formed by trout liver microsomes are phenols, dihydrodiols, and quinones. The production of dihydrodiols implies the formation of epoxides. The mutagenicity and/or carcinogenicity of these remains to be investigated in detail. In the thin-layer chromatography separations, some metabolites appeared at the origin. These may be polyhydroxylated products, which may mean that epoxides of previously formed dihydrodiols are produced. Certain isomers of these are known to be strongly mutagenic (20). We have shown that trout liver microsomes in fact catalyze the formation of mutagenic metabolites of BP. Also, in the course of in vitro metabolism of BP there occurs covalent binding of BP to macromolecules.6

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