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BENZO[*a*]PYRENE-HYDROXYLASE CATALYZED BY PURIFIED ISOZYMES OF CYTOCHROME P-450 FROM β-NAPHTHOFLAVONE-FED RAINBOW TROUT

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Abstract—We have purified five isozymes of liver microsomal (LM) P-450 from β-naphthoflavone-fed rainbow trout. Four forms (LM_3, LM_1, LM_{4a} and LM_x) were resolved on DEAE-Sepharose. Chromatography on hydroxylapatite further resolved LM_x into two components, LM_2 and LM_{4b}. This latter form, obtained in highest yield (5%), had an apparent minimum molecular weight (M_r), as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), of 58,000, a specific content of 11.9 nmoles/mg, a λ_{max} in the carbon monoxide-ligated, reduced difference spectrum of 447.0 nm, and was active towards benzo[*a*]pyrene in a reconstituted system. A second form, LM_{4a}, obtained in a final yield of 2%, had a specific content of 10.3 and was indistinguishable from Lm_{4b} by M_r, λ_{max}, or activity towards benzo[*a*]pyrene. Form LM_2 (2% yield) had a specific content of 10.8, a M_r of 54,000, a λ_{max} of 449.5 nm, and was not effective in reconstitution of benzo[*a*]pyrene-hydroxylase. In addition, two other forms with lower specific contents were obtained, LM_1 and LM_3. Neither LM_1 nor LM_3 was active towards benzo[*a*]pyrene. The properties of LM_2, LM_{4a} and LM_{4b} were further examined with the aid of antibodies prepared from rabbits. Antibodies to LM_{4a} and LM_{4b} each cross-reacted with the other antigen and formed lines of identity on Ouchterlony plates, and both IgGs exhibited some cross-reaction to P-448 from rat. Neither antibody cross-reacted with trout LM_2, and LM_2-IgG did not cross-react with any other purified P-450. Benzo[*a*]pyrene-hydroxylase, catalyzed by either LM_{4a} or LM_{4b}, was inhibited by LM_{4b}-IgG but not by LM_{4a}-IgG, suggesting that these antibodies recognize different antigenic sites. Further comparison of LM_{4a} and LM_{4b} by amino acid composition, peptide mapping, kinetic properties, sensitivity to α-naphthoflavone, and regioselectivity towards benzo[*a*]-pyrene-dihydrodiol formation indicates that these forms are highly similar in structure and function.

Cytochrome P-450 from mammals has been shown to exist as a family of isozymes differing in substrate specificity and regioselectivity, molecular weight, spectral properties, amino acid composition and partial sequences, sensitivity to *in vitro* inhibitors, peptide patterns following proteolysis, levels following treatment with various inducers, and immunochemical properties [1–3]. Characterization and comparison of these various forms have been made possible with techniques for solubilizing P-450 from microsomal membranes with detergent without loss of catalytic activity. The most widely studied mammals, rat and rabbit, probably have five to seven distinct liver microsomal P-450 isozymes which have been purified and thoroughly characterized [13]. However, as old techniques are improved and new purification methods [4–6] developed, ever increasing numbers of similar P-450 forms are being resolved [7–10].

The liver microsomal P-450-dependent MFO‡ system of fish has been found to have a number of interesting general properties including a lower temperature optimum [11] and a lower P-450 specific content and activity [12, 13] than their mammalian counterparts. Furthermore, no species of fish has been shown to be responsive to PB or "PB-type" inducers such as dichlorodiphenyltrichloroethane (DDT), non-coplanar PCB or PBB isomers, mirex or kepone [14–19], whereas pretreatment with 3-MC, and similar inducers such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), BNF, coplanar PCB and PBB isomers, produces significant increases in P-450 levels and Ah-associated activities such as BP hydroxylase and ethoxyresofurin-*O*-deethylase [18–21]. The characteristic hypochromic shift of the λ_{max} of the CO-reduced difference spectrum to 447–448 nm, seen in mammals, has not been observed in most fish studies [22, 23]. Investigations with microsomes from untreated or induced trout suggest that the P-450 in both cases appears similar to 3-MC-induced "P-448(s)" from mammals [24–25]. The level(s) of this "P-448(s)" in fish populations, as determined by BP hydroxylase activity, varies widely, even between strains [26]. How much of this variation is genetically determined and how much is

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‡ Abbreviations: MFO, mixed-function oxidase; PB, phenobarbital; PCB (PBB), polychlorinated (brominated) biphenyls; BP, benzo[*a*]pyrene; 3-MC, 3-methylcholanthrene; BNF, β-naphthoflavone; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; DLPC, dilauroylphosphatidylcholine; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; M_r apparent monomeric molecular weight; ANF, α-naphthoflavone; and PMSF, phenylmethylsulfonyl fluoride.

due to exposure to environmental pollutants is a problem that is of great importance from a comparative, evolutionary and, perhaps, ecological viewpoint.

Characterization of the properties of fish P-450s has lagged considerably behind the great number of sophisticated studies in mammals, due, in part, to the lack of purified fish isozymes. In 1978, Bend and coworkers [27] had the first success with a partial purification of a "P-451" and a "P-447" from hepatic microsomes of little skate that had been pretreated with benzo[*a*]anthracene. Recently, Stegeman and coworkers [28] have purified multiple isozymes of P-450 from BNF-pretreated scup. We have developed a procedure for purifying multiple forms of P-450 from BNF-pretreated rainbow trout [29]. Comparison of the properties of the main trout isozyme with rat P-448 and P-450 demonstrated that the trout hemoprotein was quite similar to rat P-448 [30]. In this paper we examined the properties of five isozymes of P-450 purified from BNF-fed rainbow trout and found evidence for the existence of two very similar isozymes effective in hydroxylation or epoxidation of BP.

MATERIALS AND METHODS

Pretreatment of trout with BNF and purification of P-450. Rainbow trout of the Mt. Shasta strain (average weight, 275 g) were induced by feeding *ad lib.* a standard diet [31] containing 500 ppm BNF. The fish were fed this diet for 2 weeks, starved for 4 days, and then killed by a blow to the head. Livers from eighty fish were pooled, the microsomes were prepared and solubilized with CHAPS, and the cytochromes P-450 were purified as previously described [29].

Microsomes were resuspended (10 mg ml) in 0.1 M potassium phosphate, pH 7.25, 20% glycerol, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF). CHAPS were added dropwise to a final concentration of 1%. After stirring at 4° for 30 min, the mixture was centrifuged at 100,000*g* for 60 min. The supernatant fraction was diluted 10-fold with the solubilization buffer (without CHAPS) and added directly onto a 2 × 20 cm tryptamine Sepharose 4B column (prepared as described in Ref. 29). The column was washed with four column volumes of 0.1 M potassium phosphate, pH 7.25, 20% glycerol, 1 mM EDTA, 0.1 mM PMSF, 0.5% CHAPS and 1% Lubrol PX, and the P-450 was eluted with the same buffer containing 0.5% sodium cholate (in place of CHAPS) and 0.2% Lubrol PX. Fractions containing a high A_{417}/A_{280} ratio were pooled, concentrated (PM 30), and dialyzed against 20% glycerol, 1 mM EDTA, 0.1 mM PMSF for 2 hr, followed by dialysis overnight against 10 mM potassium phosphate, pH 7.7, 20% glycerol, 1 mM EDTA, 0.2% cholate, 0.1% Lubrol PX, 0.1 mM PMSF (buffer A). The sample was loaded onto a DEAE-Sepharose column which was then washed with buffer A and eluted with a linear gradient of NaCl in buffer A. Fractions with high A_{417}/A_{280} ratios from each DEAE peak (LM_3, LM_1, LM_{4a} and LM_x) were pooled separately, treated with Bio-Beads SM-2, concentrated and dialyzed overnight against buffer A but with the pH adjusted to 7.25 and the concentration of EDTA lowered to 0.1 mM (buffer B).

Following dialysis, the samples were loaded onto hydroxylapatite columns (1.2 × 10 cm) which were then washed with buffer B followed by elution with a linear gradient of 10–125 mM potassium phosphate in buffer B. Fractions with an A_{417}/A_{280} ratio of 1 or higher were pooled, treated with Bio-Beads, concentrated, dialyzed twice against 10 mM Tris–HCl, pH 7.4, 20% glycerol, 0.1 mM EDTA, 0.1 mM PMSF, and stored in small aliquots at −70°.

Purification of rat enzymes. P-450 was purified from PB-treated rats and P-448 from BNF-treated rats as described by Guengerich and Martin [32] to specific contents of 13.8 and 11.6 nmoles/mg respectively. Epoxide hydrolase (sp. act. 442 nmoles styrene oxide hydrated · min^{-1} · mg^{-1}) and NADPH-cytochrome P-450 reductase (sp. act. 32 μmoles cytochrome *c* reduced · min^{-1} · mg^{-1}) were purified from rats pretreated with Arochlor 1254 [32].

Reconstitution of P-450 *activity.* P-450 (typically 0.1 to 0.2 nmole) was preincubated for 10 min at room temperature with a 2-fold molar excess of NADPH-P-450 reductase and 20 μg of DLPC. Following addition of buffer to 1 ml, substrate was added (in acetone to 60 μM for [^{14}C]BP and in methanol to 20 μM for [^{14}C]acetanilide), and the reaction was initiated with the addition of NADPH to a final concentration of 1.0 mM. The reaction was allowed to proceed for 30 min at 30°. BP hydroxylase was determined with [^{14}C-7,10]BP as substrate (Amersham) which had been diluted with unlabeled BP (Aldrich) to a specific activity of 2–7 μCi/μmole and purified by TLC. Quantitation of BP hydroxylase was performed by a radiometric procedure [33] and the BP metabolite profile by HPLC [34]. Individual [^{14}C]BP metabolites were identified by coelution with unlabeled BP metabolite standards provided by Dr. Donald M. Jerina of NIH.

[^{14}C-G]Acetanilide (Pathfinder, sp. act. 1.6 μCi/μmole) was purified by TLC before use. Acetanilide-4-hydroxylase was determined by HPLC [35] with [^{14}C]-4-hydroxyacetanilide identified by coelution with unlabeled 4-hydroxyacetanilide (Sigma).

Analysis of amino acid composition and peptide patterns following proteolysis. Trout LM_2, LM_{4a} and LM_{4b} (200 μg each) were dialyzed against two changes of distilled water to remove glycerol. The P-450s were then hydrolyzed for 22 hr at 100° in 6 M HCl in sealed evacuated tubes. The hydrolysates were analyzed in a Beckman model 120 B amino acid analyzer updated to a 6 mm single column system. Tryptophan was estimated with a fluormetric procedure [36]. Peptide maps on SDS-PAGE of LM_2, LM_{4a} and LM_{4b} were obtained using the procedure of Cleveland *et al.* [37] as modified by Guengerich [38]. The enzymes used for proteolysis were papain, α-chymotrypsin (Sigma), trypsin (Grand Island Biological Co.) and *Staphylococcus aureus* V8 proteases (Miles Laboratories, Ltd.).

Preparation of antibodies. Antibodies were raised to LM_2, LM_{4a} and LM_{4b} with the injection schedule of Thomas *et al.* [39]. Male New Zealand white rabbits were injected intradermally at numerous sites along the shaved back with 200 μg of antigen which had been diluted first with saline to 1 ml followed by

Freund's complete adjuvant to 2 ml. After 6 weeks, the rabbits were injected again, as above, but with 40 μg of antigen in Freund's incomplete adjuvant. Four weeks later, 40 μg of antigen (without adjuvant) was injected intravenously into an ear vein. One week later, blood was collected from the ear vein (30–40 ml). Rabbits were bled approximately every 10 days. For continued production of antibody, the rabbits were boosted with intramuscular injections of 20 μg of antigen when the antiserum titer declined.

The rabbit antisera were treated as described by Kaminsky *et al.* [40], and the IgG fractions purified by ammonium sulfate precipitation followed by DEAE-Sepharose [40]. Antibodies were stored at −20° in 20 mM potassium phosphate, pH 8.0, at a concentration of 50 mg/ml.

Antibody titer and immunological cross-reactivity were assayed with Ouchterlony plates which were prepared and utilized as described by Guengerich *et al.* [41].

Other assays. Cytochrome P-450 was assayed by the method of Estabrook *et al.* [42] and NADPH–P-450 reductase by the reduction of cytochrome *c* [43] at 20°. SDS-PAGE [44] was done on slab gels (15 cm long, 1.5 mm thick) with a 9% separating gel. The gels were run under constant power (3–5 hr at 20 W/gel) with cooling. Proteins were fixed and stained by shaking overnight in 45% ethanol, 10% acetic acid, 0.1% Coomassie brilliant blue R-250 (Sigma) and destained with 10% ethanol, 7.5% acetic acid. Molecular weights were calculated by comparing the relative mobility of unknowns to the log of the molecular weights of a set of protein standards (bovine serum albumin, 66,000; glutamate dehydrogenase, 53,000; ovalbumin, 45,000; and aldolase, 40,000). All equipment and chemicals used for SDS-PAGE were from Bio-Rad. Proteins were assayed [45] with bovine serum albumin as the standard. Epoxide hydrolase was assayed using [7-^{3}H] styrene oxide (Amersham) as substrate [46].

Proteins were concentrated with ultrafiltration cells and PM-30 or YM-30 Diaflo membranes (Amicon).

Lubrol PX (Sigma), used for P–450 purification, was removed from the final hydroxylapatite fractions by adsorption to Bio-Beads SM-2 (Bio-Rad) prior to concentration and final dialysis. In addition, with some purified trout P-450 fractions, it was found that heme loss could be reduced if pooled fractions from DEAE-Sepharose were treated with Bio-Beads before being concentrated prior to hydroxylapatite chromatography.

Other materials. All other chemicals or column materials were prepared in the same manner or obtained from the same source as described previously [29].

Enzyme nomenclature. In an attempt to minimize confusion, we have adopted the nomenclature system employed for purified rabbit P-450s. Therefore, the purified trout forms are designated trout LM (liver microsomal) 1, 2, 3, 4a and 4b according to increasing electrophoretic mobilities on SDS-PAGE. According to this system, the main trout isozyme (f) previously purified [29] would correspond to trout LM_{4b}.

RESULTS

Purification of trout. P-450s. Treatment of microsomes with the zwitterionic detergent CHAPS (1%) solubilized 96% of the P-450 with little or no conversion to P-420. Total P-450 was eluted from tryptamine-Sepharose as described previously [29], except that the first two washes were eliminated. A minimum of four P-450s (LM_3, LM_1, LM_{4a} and LM_x) were resolved by elution from DEAE-Sepharose (Fig. 1). Subsequent chromatography on hydroxylapatite yielded single P-450 peaks for LM_3 and LM_1 and for LM_{4a} (Fig. 2, top), but LM_x was further resolved into two fractions, LM_2 and LM_{4b} (Fig. 2, bottom). The specific content and yield for each fraction are given in Table 1. Isozymes LM_2, and LM_{4a} and LM_{4b} had specific contents of 10.8, 10.3 and 11.9 nmoles/mg respectively.

Molecular weight, Co-reduced λ_{max} and BP-hydroxylase activity of purified trout P-450. The molecular weight and relative degree of purity of each fraction were examined by SDS-PAGE and compared to the microsomes from which they were purified and to microsomes from untreated rainbow trout (Fig. 3) to attempt to identify constitutive and BNF-induced P-450 isozymes. Form LM_1 (eluted as a shoulder on LM_3) had a molecular weight of 50,000, a λ_{max} of 449.0 nm, and a specific content of 6.8 and yield of 1.6% (Table 1). In a reconstituted system, isozyme LM_1 had no detectable activity towards BP (Table 1). The relatively low specific content was not consistent with the high degree of apparent purity as judged by SDS-PAGE (Fig. 3). Only one band was observed in the molecular weight range of P-450, and only two other faint bands were apparent. This same observation holds true for LM_3, which had the lowest (3.0) specific content and yield (1.2%) but

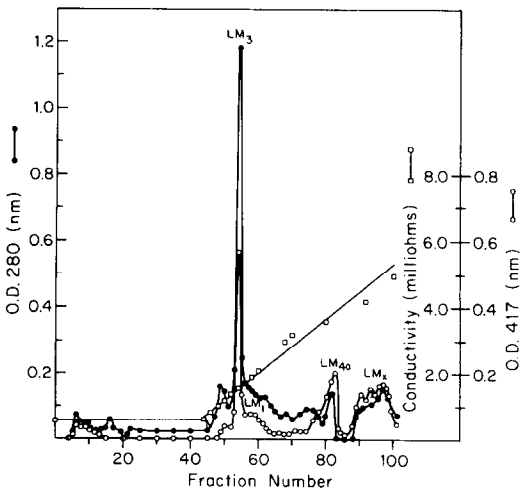

Fig. 1. Resolution of trout P-450s by elution from DEAE-Sepharose. Approximately 500 nmoles P-450 (250 mg protein, partially purified by elution from tryptamine-Sepharose) was loaded onto a DEAE-Sepharose column (2 × 50 cm) previously equilibrated with buffer A. The column was washed with 500 ml of buffer A and eluted with a linear gradient of 10–125 mM NaCl (500 ml) at a flow rate of 30 ml/hr.

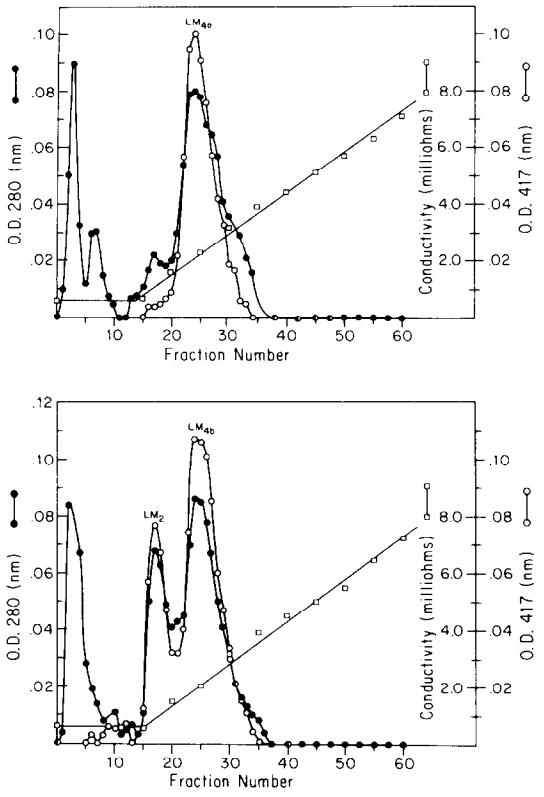

Fig. 2. Top: Hydroxylapatite chromatography of DEAE peak LM_{4a}. Fractions which eluted from DEAE as peak LM_{4a} were combined, treated with Bio-Beads SM-2, concentrated, and dialyzed with buffer B. The sample was loaded onto hydroxylapatite (1.2 × 10 cm), washed with 100 ml of buffer B, and eluted with a linear gradient (500 ml) of 10–125 mM potassium phosphate in buffer B at a flow rate of 20 ml/min. Bottom: Resolution of DEAE-Sepharose peak LM_4 into two fractions (LM_2 and LM_{4b}) on hydroxylapatite. Fractions from DEAE-Sepharose labeled as peak LM_x were combined and chromatographed as described above. Fractions containing LM_2 and LM_{4b} (A_{417}/A_{280} at least 1) were treated as above for LM_{4a}.

appeared pure on SDS-PAGE (Fig. 3). Neither isozyme had detectable epoxide hydrolase activity but it is possible that another microsomal protein, such as UDP-glucuronyl transferase with a molecular weight in the P-450 range, could be present. Alternatively, the low specific content could be due to a higher loss of heme during purification of these isozymes. The calculated M_r for LM_3 was 56,500, and this isozyme also exhibited no detectable BP hydroxylase activities (Table 1).

Isozyme LM_2 (specific content 10.8 and yield 1.9%) appeared homogeneous on SDS-PAGE, exhibiting a M_r of 54,000 and a λ_{max} of 449.5 nm. This form also showed no detectable BP hydroxylase activities but has been found previously to be very active towards aflatoxin B_1 [47].

Isozymes LM_{4a} and LM_{4b}, although resolved on DEAE-Sepharose (Fig. 1), appeared to have identical M_r values (58,000) on SDS-PAGE (Fig. 3), and both appeared as doublets which were otherwise electrophoretically homogeneous. Isozyme LM_{4b} was obtained in highest yield (5.3%) and had the highest specific content (11.9), while the specific content and yield of LM_{4a} were 10.3 and 2% respectively. Both LM_{4a} and LM_{4b} had the same λ_{max} of 447.0 nm and gave very similar turnover numbers with BP (Table 1). The additive BP-hydroxylase turnover numbers of LM_{4a} and LM_{4b} (0.86) were less than the turnover number with microsomes from BNF-treated trout (1.47) (Table 1). However, the former number was obtained in a reconstitution system which contained no added epoxide hydrolase. Inclusion of a molar ratio of purified rat epoxide hydrolase to trout LM_{4a} or LM_{4b} in the reconstitution system of 1:1 increased the turnover numbers to 0.87 and 0.74 respectively. This additive turnover (1.61) slightly exceeded that seen in microsomes.

These results obtained with LM_{4a} and LM_{4b} are similar to those obtained previously [29] in which we purified, from BNF-treated trout, two "P–448s" effective in reconstituting BP hydroxylase, a major isozyme (f) and a minor isozyme (g). Subsequently

Table 1. Properties of P-450 fractions purified from BNF-fed trout

Fraction	Specific content* (nmoles/mg)	Yield† (%)	λ_{max}‡ (nm)	M_r§ (10^{-3})	Benzo[*a*]pyrene hydroxylase‖ [nmoles·min^{-1}·(nmole P-450)]	Acetanilide‖
Microsomes	0.60	100	448.0		1.47	
LM_3	3.0	1.2	447.5	56.5	ND¶	
LM_1	6.8	1.6	449.0	50.0	ND	
LM_{4a}	10.3	2.0	447.0	58.0	0.44	0.55
LM_2	10.8	1.9	449.5	54.0	ND	
LM_{4b}	11.9	5.3	447.0	58.0	0.42	0.53

* Specific content is given as nmoles P-450 per mg protein [42] from fractions following hydroxylapatite chromatography, detergent removal, and dialysis.

† Percent yield was calculated from the amount of total microsomal P-450 present prior to solubilization.

‡ The λ_{max} of the Soret peak in the CO-reduced vs CO spectrum was obtained using a Cary 219 spectrophotometer, with an accuracy of no less than ±0.5 nm.

§ M_r was calculated from SDS-PAGE gels as described in Materials and Methods.

‖ Reconstitution of trout P-450s and measurement of benzo[*a*]pyrene and acetanilide hydroxylase were described in Materials and Methods.

¶ ND, not detectable.

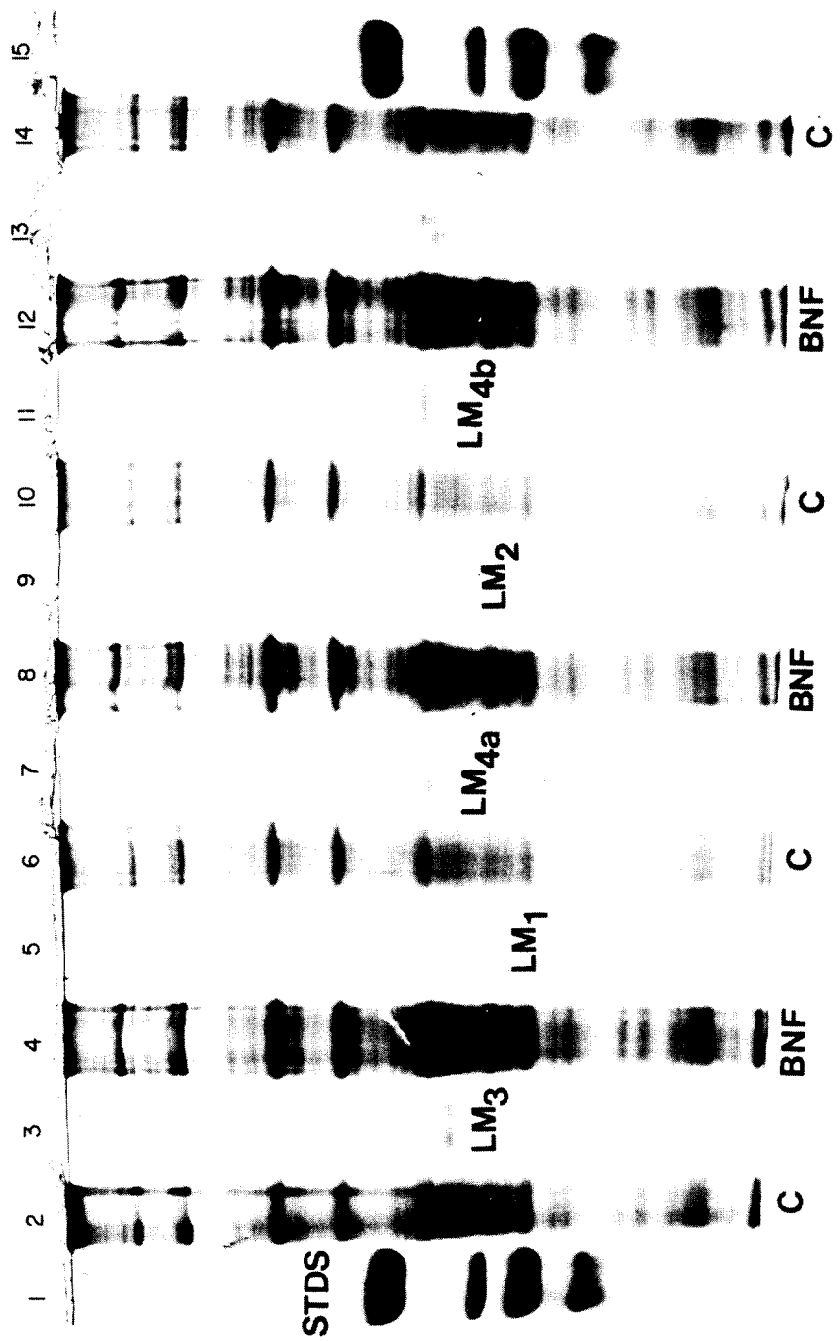

Fig. 3. SDS-PAGE of purified trout P-450s and microsomes from untreated and BNF-treated trout. Samples were prepared as described by Laemmli [44], and SDS-PAGE analysis was performed as described in Materials and Methods. Lanes 1 and 15 contained protein standards (5 μg bovine serum albumin and ovalbumin, 2 μg glutamate dehydrogenase and aldolase); lanes 2, 6, 10 and 14 contained 50 μg of microsomal protein from untreated rainbow trout; lanes 4, 8 and 12 were 50 μg of microsomal protein from BNF-treated trout; purified trout P-450s (1 μg each) were in lanes 3 (LM_3), 5 (LM_1), 7 (LM_{4a}), 9 (LM_2) and 11 (LM_{4b}). Lane 13 contained all five purified forms (0.5 μg each).

[30], we compared the properties of the major trout P-448 (isozyme f) to rat P-448 and P-450 and concluded that it was a P–448 type of isozyme. Due to the rather limited amount of the minor isozyme (g), it was not possible to confirm whether f (LM_{4b}) and g (LM_{4a}) were distinct isozymes. Part of the aim of this study was to obtain enough of each isozyme to address this question. In the results that follow, we further compared LM_{4a}, LM_{4b} and, in some cases, LM_2 by a variety of techniques including cross-reactivity to rabbit antibodies raised against each antigen.

Properties of BP hydroxylase catalyzed by reconstituted LM_{4a} and LM_{4b}. Isozymes LM_{4a} and LM_{4b}, the only purified trout isozymes capable of reconstituting BP hydroxylase, were compared with respect to kinetic properties, regioselectivity towards ANF or antibodies.

Similarities between reconstituted BP hydroxylase catalyzed by trout LM_{4a} and LM_{4b} were examined by determination of the K_m and V_{max} values from Lineweaver–Burk plots (data not shown). Both forms had very similar K_m values (10.1 and 7.7 μM for LM_{4b} and LM_{4a} respectively) and V_{max} values (389 and 354 pmoles·min^{-1}·nmol^{-1} for LM_{4b} and LM_{4a} respectively). Therefore, LM_{4a} and LM_{4b} BP hydroxylase could not be distinguished kinetically.

The [^{14}C]BP metabolite profiles with LM_{4a} and LM_{4b}, as determined by HPLC (Fig. 4), were very similar. In the absence of added rat epoxide hydrolase (Fig. 4, top, and Table 2), very little BP-dihydrodiol formation was observed. The metabolite profile with LM_{4b} differed from LM_{4a} in that an unknown radioactive peak, eluting between the dihydrodiols (DHD) and quinones, was observed with LM_{4b}. Addition of increasing amounts of purified rat epoxide hydrolase (Fig. 4, bottom, and Table 2) to both LM_{4a} and LM_{4b} increased total metabolite formation about 2-fold and produced increasing proportions of dihydrodiols and decreases in the Phenol I (containing 9-OH-BP) and Phenol II (containing 3-OH-BP) fractions. Increases in dihydrodiol formation were specific for the non-K region (7,8- and 9,10-dihydrodiol) of BP. Epoxide hydrolase increased formation of the K-region [4, 5] dihydrodiol only slightly. These results are consistent with the low amount of BP-4,5-dihydrodiol formation by BNF-rainbow trout microsomes [30]. Decreases in BP-phenols, concurrent with increases in dihydrodiols, indicate that a large portion of phenols generated by trout LM_{4a} and LM_{4b} do so via epoxide intermediates. The decrease in the Phenol II fraction was probably due to reduced formation of 7-OH-BP, which co-elutes with 3-OH-BP with this HPLC system.

Addition of ANF to either LM_{4a} or LM_{4b} produced a concentration-dependent inhibition of reconstituted BP hydroxylase (Fig. 5). Isozyme LM_{4a} appeared slightly more sensitive to ANF at concentrations between 0.5 and 1.0 μM, whereas at concentrations between 5 and 10 μM LM_{4b} was inhibited significantly more than LM_{4a}. Concentrations of 100 mM or greater of ANF reduced the microsomal BP-hydroxylase of BNF-treated trout to 5-10% that measured with no inhibitor present (data not shown).

Incubation of either LM_{4b} or LM_{4a} with LM_{4b}-IgG produced a concentration-dependent decrease in BP hydroxylase (Fig. 6). The LM_{4b}-IgG was more effective in inhibiting its own antigen up to levels of IgG which produced about 90% inhibition (10 mg IgG/nmole). Antibody to LM_{4a} (LM_{4a}-IgG) was not effective in inhibiting BP hydroxylase catalyzed by either LM_{4a} or LM_{4b}. These data suggest that LM_{4b}-IgG binds to an antigenic site on both LM_{4b} and LM_{4a} which affects catalysis, whereas binding of LM_{4a}-IgG occurs at a site other than the active site of the enzyme. Furthermore, the greater degree of sensitivity of LM_{4b}, compared to LM_{4a}, to inhibition by LM_{4b}-IgG suggests that these are distinct isozymes. Incubation of either LM_{4a} or LM_{4b} with high levels of LM_2-IgG had no effect on BP hydroxylase (data not shown). The ability of LM_{4a}-IgG to inhibit microsomal BP hydroxylase has not been tested.

Analysis of cross-reactivity on Ouchterlony plates. Double diffusion immunoprecipitin analysis on Ouchterlony plates showed that, besides precipitating its own antigen, LM_{4b}-IgG cross-reacted with LM_{4a} with a line of identity with the precipitated LM_{4b} (Fig. 7, left). Rat P-448, purified from hepatic microsomes of BNF-treated rats, showed a faint cross-reaction which did not have a line of identity to trout LM_{4b}. Neither trout LM_2 nor rat P-450 (from PB-treated rat) cross-reacted with LM_{4b}-IgG. Results identical to those shown in Fig. 7, left, were obtained if LM_{4a}-IgG replaced LM_{4b}-IgG as the antibody. The antibody LM_2-IgG was only effective in precipitating LM_2 and did not cross-react with any other trout or rat isozyme (Fig. 7, right).

Amino acid compositions of LM_2, LM_{4a} and LM_{4b}. The amino acid compositions of LM_{4a} and LM_{4b} were quite similar (Table 3), as might be expected considering the results presented above. Differences between these two forms were noted, however, (difference index [48] of 3.8), with LM_{4a} having relatively lower amounts of tyrosine and phenylalanine compared to LM_{4b}. Both trout P-448 forms (LM_{4a} and LM_{4b}) could readily be distinguished (difference index of 5.6 to 7.3) from the trout P-450 (LM_2) isozyme which had proportionally higher amounts of threonine and glycine and lower amounts of isoleucine (Table 3). The percentage of hydrophobic residues was 40.5% for both LM_{4a} and LM_{4b} and 38.3% for LM_2.

DISCUSSION

Purification of multiple P-450s from hepatic microsomes of BNF-fed trout produced a minimum of five isozymes. Two of these isozymes (LM_1 and LM_3), although appearing fairly pure on SDS-PAGE, had relatively low specific contents, perhaps due to heme loss during purification. Neither isozyme was effective in catalyzing BP hydroxylase.

Trout LM_2 appeared homogeneous on SDS with an M_r of 54,000, and a λ_{max} near 450 nm. This isozyme was ineffective in reconstituting BP hydroxylase. Trout LM_2 is similar to P-450 from PB-treated rat with respect to the activation of aflatoxin B_1 to the carcinogenic 2,3-epoxide [47, 49]. In fact, the trout isozyme is at least an order of magnitude more effective than rat P-450 in catalyzing this conversion, perhaps explaining the great susceptibility of rainbow

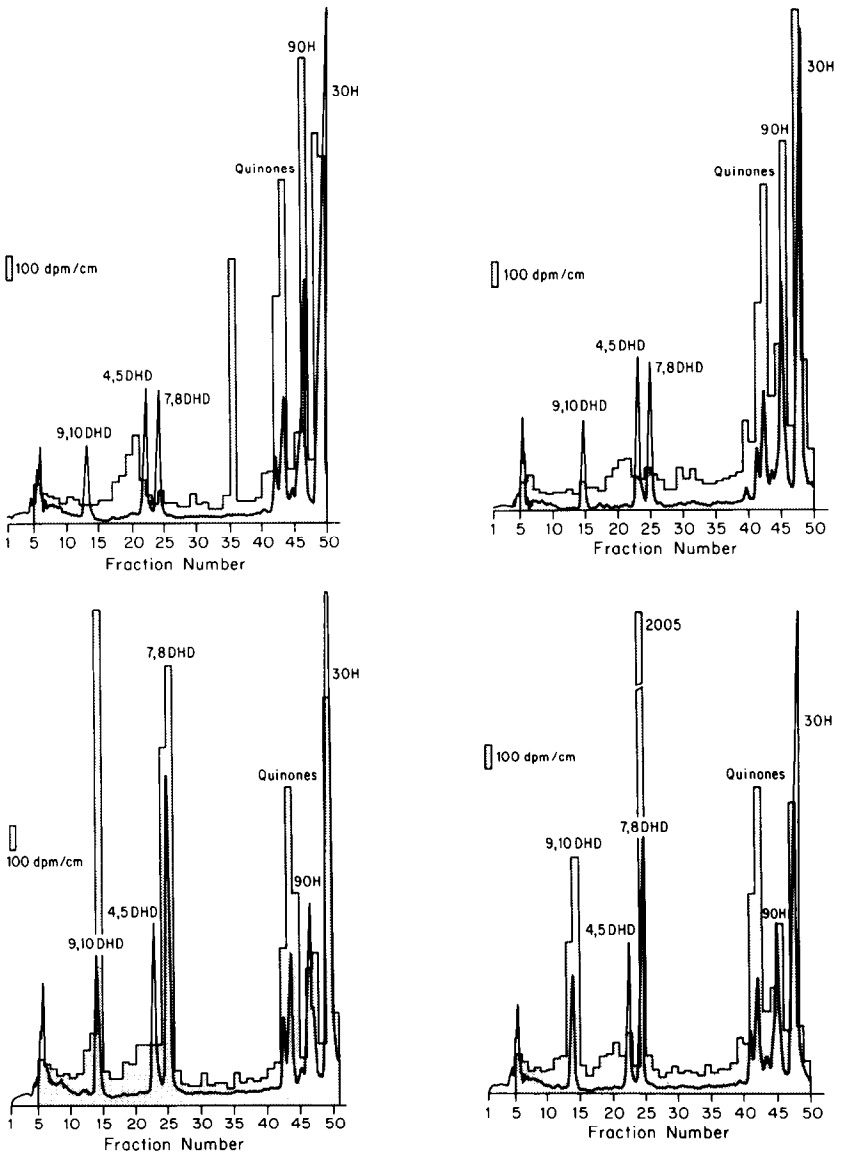

Fig. 4. HPLC metabolite profile of BP by LM_{4a} and LM_{4b} reconstituted with (bottom) or without (top) epoxide hydrolase. Top: trout LM_{4b} (left) and LM_{4a} (right) were reconstituted with rat NADPH–P-450 reductase, DLPC and [^{14}C]BP as described in Materials and Methods. The reaction was terminated with CH_2Cl_2:MeOH (2:1), spiked with nonlabeled BP metabolite standards and extracted, and the organic layer was evaporated with N_2. The residue was redissolved in 100 μl methanol, an aliquot was injected into a Zorbax ODS (4.6 mm i.d. × 25 cm) column, and the metabolites were eluted with a gradient of acetonitrile (40–85%) in water at 40° with a flow rate of 2 ml/min. Total metabolites were monitored at 254 nm (solid line) with an Altex u.v. detector, and radioactivity was assayed by collecting 0.5-min fractions directly into scintillation vials and counting. Bottom: Trout LM_{4b} (left) and LM_{4a} (right) BP-hydroxylase were reconstituted, and the [^{14}C]BP metabolite profile was analyzed as above, except that a 10-fold molar excess of purified rat epoxide hydrolase was included in the reconstitution system.

trout to aflatoxin B_1-induced hepatocarcinogenesis [31]. Antibodies to trout LM_2 did not cross-react with rat P-450 and the amino acid composition differed widely (difference index of 10.2) from a previously published composition of rat P-450 [1].

Trout LM_{4a} and LM_{4b} were indistinguishable with respect to M_r, λ_{max} or activity towards BP and acetanilide (Table 1). Evidence has been presented previously that mammalian species respond to BNF or 3-MC with the synthesis of at least two P-450 isozymes [50–53]. Nebert and Negishi [54], postulate that a major isozyme of BNF-inducible P-450 (P-448), having the maximum blue shift in the λ_{max}, represents a protein product of a structural gene common to rabbit, rat, mouse and fish. This P-448 can be distinguished from another BNF-inducible P-450 (P_1-450), in that P-448 has a higher turnover with acetanilide and a lower turnover with BP, as compared to P_1-450. Other differences are seen in the peptide patterns following proteolysis, immuno-

Table 2. [^{14}C]BP metabolite profile of reconstituted trout LM_{4a} and LM_{4b} with increasing amounts of epoxide hydrolase

Molar ratio of EH/P-448*	Dihydrodiols 9, 10	Dihydrodiols 4, 5	Dihydrodiols 7, 8	Quinones†	Phenol I (9-OH)	Phenol II (3-OH)
			LM_{4a}			
0	1.6	1.9	1.6	24.0	31.4	39.7
0.1	1.7	2.4	3.0	30.5	29.7	32.6
1	4.9	3.2	13.3	28.0	25.4	25.2
10	17.4	3.7	25.3	24.7	13.2	15.6
			LM_{4b}			
0	1.4	2.6	1.9	28.5	28.5	37.1
0.1	1.6	1.6	2.8	29.0	29.0	35.8
1	5.9	3.4	14.0	25.6	23.9	27.2
10	20.0	3.7	27.9	22.4	10.1	15.9

* LM_{4a} or LM_{4b} (0.1 nmole) were reconstituted with 0, 0.01, 0.1 or 1 nmole of purified epoxide hydrolase, and the reconstituted [^{14}C]BP metabolite profile was analyzed by HPLC as described in Materials and Methods. Individual metabolites were quantitated by coelution with unlabeled standards (Fig. 5) and are from a single determination. Values are the percentage of the total metabolites coeluting with standards (80–90% of total ^{14}C).

† 1,6; 3,6; and 6,12.

logical properties, developmental stages at which they are induced, levels in untreated animals, and the presence of glucosamine [51, 52, 55].

It was not possible in this study to classify either trout LM_{4b} or LM_{4a} as a P_1-450, according to Nebert's terminology. Both isozymes had similar activity towards BP and acetanilide, and the kinetic values of K_m and V_{max} with BP for both LM_{4a} and LM_{4b} were the same. In addition, both forms showed similar regioselectivity towards BP and, in the presence of epoxide hydrolase, produced non-K region BP-dihydrodiols. The principal metabolite, when epoxide hydrolase was present, was BP-7,8-dihydrodiol, the precursor of the ultimate carcinogenic and mutagenic metabolite, BP-7,8-dihydrodiol-9,10-epoxide [56]. Microsomes from trout [57], as well as other species of fish [13], have been shown to be very

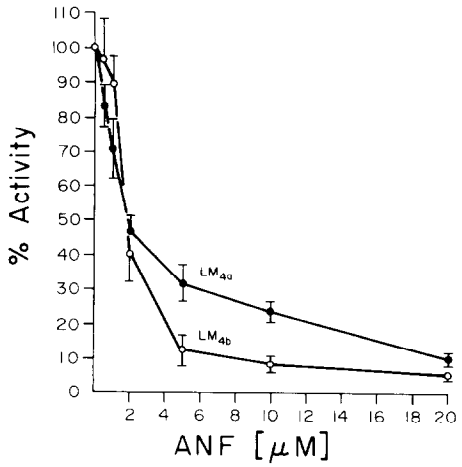

Fig. 5. Inhibition of LM_{4a} and LM_{4b} reconstituted BP-hydroxylase by ANF. BP-hydroxylase was reconstituted with trout LM_{4a} or LM_{4b}, rat NADPH–P-450 reductase and DLPC as described in Materials and Methods except that ANF was added, at different concentrations, just prior to the addition of substrate. Each point represents the mean ± S.D. of triplicates. The activity in the absence of ANF (100%) was 0.53 and 0.56 nmole · min^{-1} · mg^{-1} for LM_{4a} and LM_{4b} respectively.

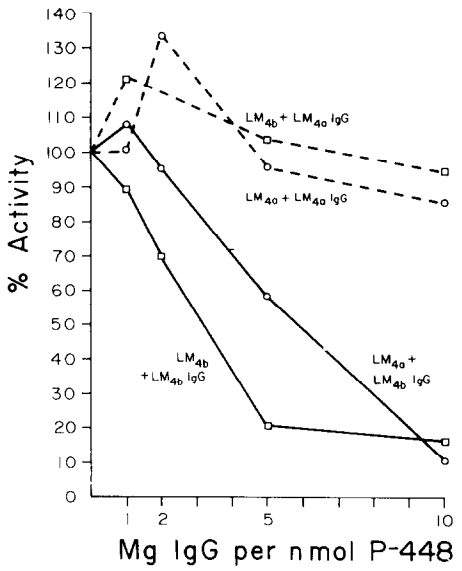

Fig. 6. Inhibition of LM_{4a} and LM_{4b} reconstitution of BP-hydroxylase by rabbit antibodies. Trout LM_{4a} (○) and LM_{4b} (□) were reconstituted as before (Fig. 6), except for a 10-min preincubation with increasing amounts of Lm_{4a}-IgG (———) or LM_{4b}-IgG (———)prior to the addition of NADPH–P-450 reductase, DPLC, substrate and NADPH. Each point represents the mean of duplicates. Activity in the absence of IgG was 0.49 and 0.43 nmole · min^{-1} · mg^{-1} for LM_{4a} and LM_{4b} respectively.

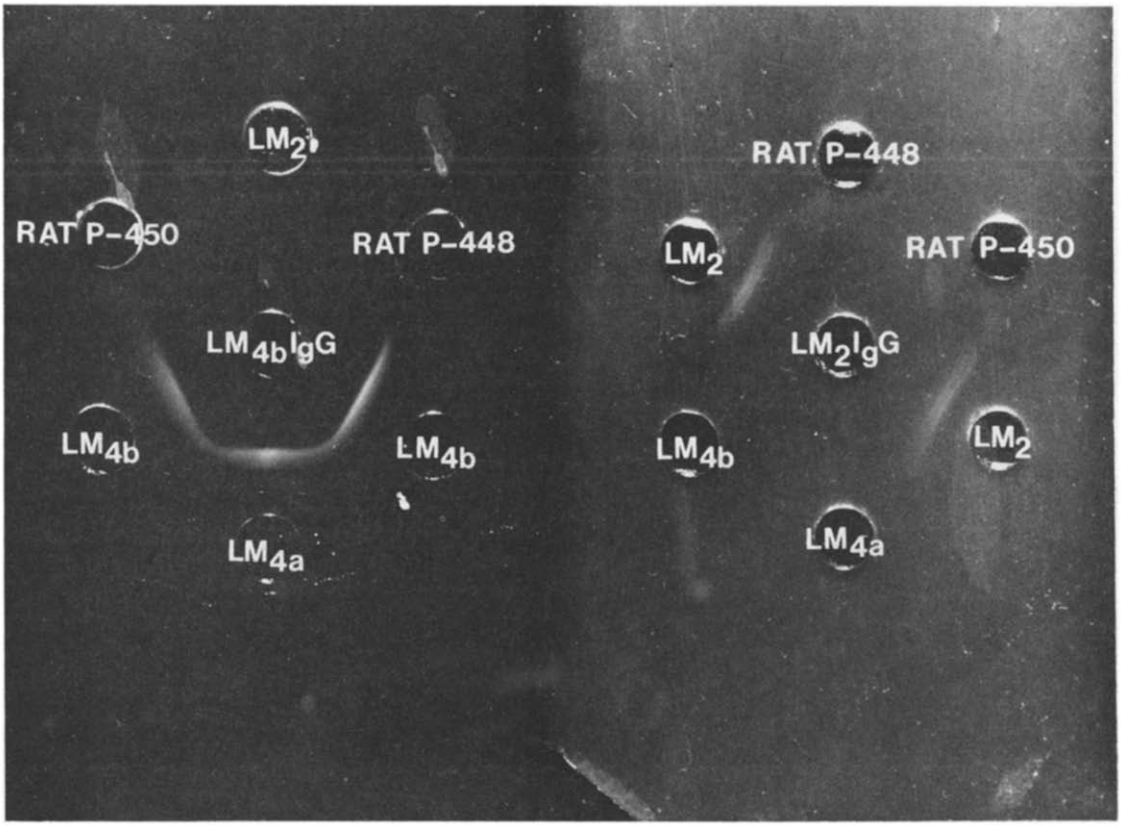

Fig. 7. Ouchterlony double precipitin analysis of trout LM_2, LM_{4a} and LM_{4b} with rabbit antibodies. Ouchterlony plates were poured and loaded as described by Guengerich *et al.* [41]. Left: The center well contained 0.5 mg of rabbit LM_{4b}-IgG. Sample wells, each containing about 5 μg of protein, were (clockwise starting at the top) LM_2, rat P-448, LM_{4b}, LM_{4a}, LM_{4b} and rat P-450. Right: The centre well contained 0.5 mg of rabbit LM_2-IgG. Sample wells (5 μg each) were (clockwise from the top) rat P-448, rat P-450, LM_2, LM_{4a}, LM_{4b} and LM_2. Each plate was developed for 24–48 hr at room temperature in a dark, humid atmosphere and then photographed directly.

effective in activating BP. This activation is maximal following BNF-type induction, but can also be quite high in fish not purposely exposed to inducers [58, 59].

Trout LM_{4a} and LM_{4b} did show slight differences in sensitivities to BP hydroxylase inhibition by ANF. Previous studies had found that microsomes from fish, like mammals, treated with BNF-type inducers were very sensitive to ANF inhibition of BP hydroxylase [13]. In untreated mammals, ANF actually activates BP hydroxylase. Similar results are obtained for some fish species but not for others [13], and it has been postulated that the degree of microsomal BP hydroxylase inhibition by ANF in fish populations could be useful as a monitor for the degree of MFO induction from exposure to environmental pollutants. In this study, only two isozymes active towards BP were isolated, and both were sensitive to ANF. Therefore, if there is a constitutive form of BP hydroxylase in trout which is insensitive to ANF, it was either absent from these BNF-treated trout or lost during purification.

Although the results from Ouchterlony plates indicated that LM_{4b}-IgG recognized identical antigenic sites on LM_{4b} and LM_{4a}, the greater sensitivity to BP hydroxylase inhibition shown by LM_{4b}, provided evidence of some structural differences between these enzymes. The LM_{4a}-IgG also appeared to recognize (as judged by Ouchterlony plates) similar or identical antigenic sites on LM_{4a} and LM_{4b}, but this site must be different from the one recognized by LM_{4b}-IgG, since this antibody was much less effective at inhibiting BP hydroxylase.

Slight differences between LM_{4a} and LM_{4b} were also suggested from the amino acid compositions, but limited proteolysis yielded peptide patterns on SDS-PAGE (data not shown) that were indistinguishable.

It has also been suggested that, in mice, P-448 is present in low concentrations in untreated animals,

Table 3. Amino acid composition of three purified P-450s from BNF-fed trout

Amino acid*	Isozymes		
	LM_{4a} (minor "P-448")	LM_2 ("P-450")	LM_{4b} (major "P-448")
Aspartate	54	48	50
Threonine	25	32	24
Serine	36	37	36
Glutamate	54	49	53
Proline	28	27	28
Glycine	42	50	43
Alanine	23	27	26
Cystine	4	3	4
Valine	34	29	32
Methionine	10	7	7
Isoleucine	32	19	29
Leucine	54	51	50
Tyrosine	13	20	19
Phenylalanine	27	29	36
Histidine	14	11	13
Lysine	30	26	30
Arginine	30	25	31
Tryptophan	3	3	3
Total	513	493	514

* The method used for amino acid analysis was as described in Materials and Methods. The molecular weight was calculated from SDS-PAGE data (Table 1). Results are given as residues per molecule and are from a single determination.

whereas P-450 is not [55]. Results using a western blotting technique indicate that there is a small band stained with LM_{4a}-IgG in microsomes from untreated trout which is increased many-fold in microsomes from BNF-treated trout [60]. Electrophoresis of purified trout forms alongside microsomes from untreated and BNF-treated trout (Fig. 3) was also not definitive in establishing which isozymes were constitutive and which were induced (or repressed) by BNF.

We have purified and characterized multiple forms of P-450 from BNF-treated rainbow trout. One isozyme (LM_2) does not appear active towards BP but has been shown previously to be very active towards aflatoxin B_1 [47]. Recent evidence suggests that LM_2 is also very effective at catalyzing the (ω-1)-hydroxylation of lauric acid [61].

Two isozymes (LM_{4a} and LM_{4b}) were found to be active towards BP and the turnover numbers obtained in a reconstitution system (in the presence of epoxide hydrolase) suggest that LM_{4a} and LM_{4b} account for the total BP metabolism of hepatic microsomes from BNF-treated rainbow trout. The physical properties and catalytic activities of LM_{4a} and LM_{4b} were quite similar. However, their resolution on DEAE-Sepharose, the differences in yield, and the amino acid composition suggest that they could be distinct isozymes. Future experiments will be designed to determine whether or not LM_{4a} and LM_{4b} are separate gene products and how these "BP hydroxylases" (as well as LM_2) are regulated as a function of age, sex, and exposure to various xenobiotics. The relative levels and activities of these various P-450 isozymes in trout liver (and other organs) will play a large role in the susceptibility of this commercially important fish to toxicity and carcinogenesis due to exposure to xenobiotics.

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