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Determination of Multiple Forms of Cytochrome P-450 in Microsomes From the Digestive Gland of Cryptochiton stelleri

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Polyclonal antibodies raised against purified trout cytochromes P-450 (P-450) LM_2 (anti-LM_2) and LM_{4b} (anti-LM_{4b}) were used in Western blot analyses with digestive gland microsomes from control and β-naphthoflavone (BNF)-treated gumboot chitons Cryptochiton stelleri. An increase and decrease in staining intensity subsequent to treatment with anti-LM_{4b} and anti-LM_2, respectively, was observed in digestive gland microsomes from BNF-treated chiton. Thus, there appears to be at least two forms of P-450 in microsomes from the digestive gland of Cryptochiton; one of which is induced by BNF and perhaps is involved in benzo(a)pyrene (BP) biotransformation, and another form which is inhibited by BNF.

Phase I biotransformation pathways have the potential to bioactivate as well as detoxify xenobiotics (1). Determination of the ratio of bioactivation to detoxication is essential in understanding the toxicity of any xenobiotic. Therefore, assuming two animals in a given habitat are exposed to the same toxicant, the animal that is equipped with enzymes which lead to detoxication of the chemical may have an advantage over the animal that either does not possess enzymes of detoxication or has enzymes which bioactivate chemicals to more toxic species. Consequently, animals with detoxication enzymatic pathways would be better equipped to survive in a chemically dynamic environment such as the marine ecosystem.

Cryptochiton stelleri has been shown to feed on algae which contain a number of relatively toxic chemicals such as bromophenols (Yates, unpublished data). In an attempt to better understand how this animal copes with the toxic chemicals that it encounters, the contribution of several xenobiotic biotransformation systems in this species was examined (2). Cytochrome P-450 was observed in microsomes from the digestive gland of Cryptochiton and benzo(a)pyrene hydroxylase (BPH) was significantly greater in animals pretreated with the cytochrome P-450 inducer, β-

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naphthoflavone (BNF) (2). This latter chemical is used as a noncarcinogenic substitute for P-450 inducing polyaromatic hydrocarbon environmental contaminants such as 3-methylcholanthrene and BP.

Since cytochrome P-450 activity appears to be inducible in most animal species, including the chiton, the purpose of this research was to determine whether multiple forms of this enzyme are present in microsomes from the digestive gland of Cryptochiton and if there is any quantitative change of these forms after treatment with BNF. Polyclonal antibodies raised against two purified forms of P-450 isolated from the livers of rainbow trout were used as probes for immunoquantitation. These two forms of P-450 in trout, LM_2 and LM_{4b} have been shown to possess different catalytic activities and substrate specificities. Isoform LM_2 has been shown to be responsible for the oxidation of aflatoxin B_1 to the more reactive epoxide while LM_{4b} has been shown to be the BNF-inducible form in trout liver (8) possibly representing a protein expressed from the P450IB gene family (9).

MATERIALS AND METHODS

Chemicals β-Naphthoflavone (BNF) was purchased from Aldrich Chemical Company (Milwaukee, WI) and [G-^{3}H]-Benzo(a)pyrene (BP) from ICN Radiochemicals (Irvine, CA). [^{14}C-7,10]-BP was obtained from Amersham (Arlington Heights, IL). All other biochemicals were purchased from Sigma Chemical Company (St. Louis, Mo).

Animal treatment and microsome preparation For induction studies, 10 animals (5 treated and 5 control), obtained by using SCUBA from subtidal rock jetties in Newport OR, were not fed for 2 weeks prior to treatment. Treated animals were dosed orally at 10 mg/kg with β-naphthoflavone (BNF) in corn oil three times a week for three weeks. Control animals received only corn oil. Animals were sacrificed by freezing at -20°C for approximately 20 min and dissected. Microsomes were prepared at 4°C by a modified procedure as described in Livingstone and Farrar (3). Tissues were placed in a 1:4 w/v ratio of 20 mM Tris-HCl buffer, pH 7.6, containing 0.5 M sucrose, 0.15 M KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT) and 0.1 mM phenylmethylsulfonylfluoride (PMSF). Homogenizations were carried out using a teflon Potter-Elvehjem homogenizer. The homogenate was centrifuged at 12,000 x g for 20 min in a Sorvall RC-2B refrigerated centrifuge. Following a second 12,000 x g spin of the supernatant, a final centrifugation at 100,000 x g for 90 min was performed. The microsomal pellet was resuspended in a storage buffer which contained 20 mM Tris-HCl pH 7.6, 20% w/v glycerol, 1 mM EDTA, 1 mM DTT, and 0.1 mM PMSF. Unused microsomes were stored at -80°C until needed.

Electrophoresis and immunoblotting Electrophoresis of microsomal proteins was performed using 8.0 % separating and 3.0 % stacking polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS) (4). Following electrophoresis, the separated proteins were transferred via electroblot to nitrocellulose sheets following a modification of the method of Towbin et al. (5) and stained by a modification of the method of Burnette (6) using polyclonal antibodies raised against purified trout cytochrome P-450s LM_2 and LM_{4b} (7). The nitrocellulose was exposed to Kodak XAR-5 X-ray film for 7 days at - 80°C. Relative quantitation of autoradiograms was performed utilizing laser densitometry (Zeineh).

RESULTS AND DISCUSSION

Microsomes from chiton digestive gland reacted with antibodies against purified trout P-450s LM_2 and LM_{4b} (Figures 1 and 2). A single band was observed to react with anti-LM_{4b} at 60,000 daltons in both control and BNF-treated samples (Figure 2). A band at 60,000 also was observed in control and BNF-treated samples stained with anti-LM_2 along with a much darker band at 54,000 daltons (Figure 1). Digestive gland microsomes from control chitons possessed greater intensities of staining with anti-LM_2 antibodies relative to BNF treated (Figure 3). Although these bands appeared to have variable intensities, each band is consistently darker than those observed from BNF-treated samples. Conversely, BNF-treated chitons possessed greater levels of reaction when stained with LM_{4b} antibodies relative to controls (Figure 3). Assuming the protein from chiton microsomes which reacted with anti-LM_{4b} is equivalent in function with trout $LM4_b$, that is, involved in biotransformation of BP, then this data suggests that multiple forms of P-450 exist in Cryptochiton and the protein at 60,000 daltons may be responsible for the augmented BPH observed in BNF-treated chitons. P-450 and associated activities were shown to be present within digestive gland microsomes from the chiton. BPH was significantly greater in BNF-treated chitons, while P-450 content was unchanged (2). A similar relationship between p-450 content and activity was observed in microsomes from rainbow trout livers (8). BNF pretreatment caused a decrease in the constitutive P-450 form LM_2 and a

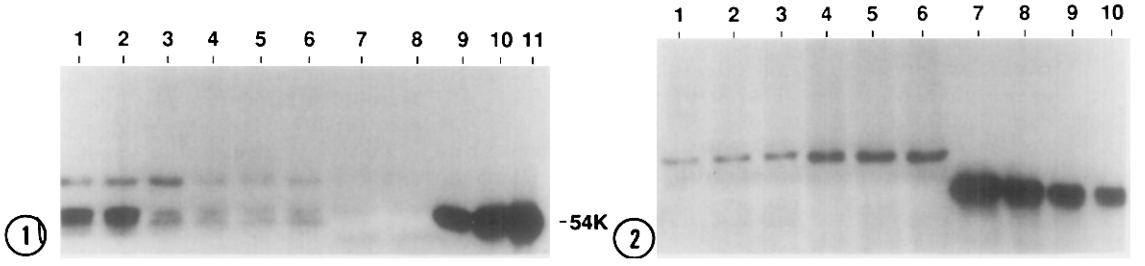

Figure 1. Western blot analysis of microsomes from the digestive glands of control and BNF-treated chiton stained with anti-trout LM_2. Lane 1-3, control microsomes, 100 µg protein; Lane 4-6, BNF microsomes, 100 µg protein; Lane 7, control cytosol, 100 µg protein; Lane 8, BNF cytosol, 100 µg protein; Lane 9-11, purified trout LM_2, 0.2, 0.5, 1.0 pmol protein.

Figure 2. Western blot analysis of microsomes from the digestive glands of control and BNF treated chiton stained with anti-trout LM_{4b}. Lane 1-3, control, 100 µg protein; Lane 4-6, BNF, 100 µg protein; Lane 7-10, purified trout LM_{4b}, 2.0, 1.0, 0.5, 0.2 pmol protein.

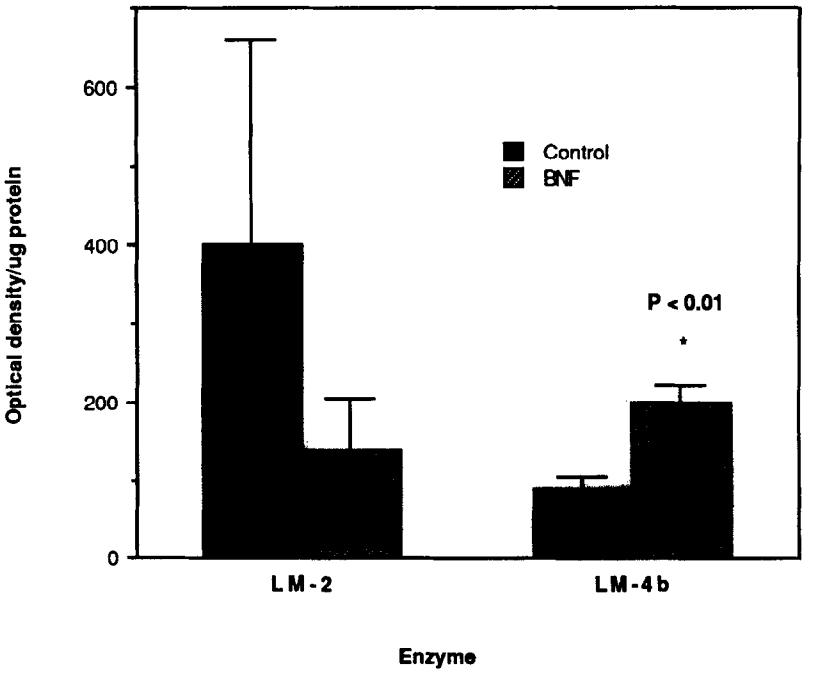

Figure 3. Relative staining intensities of microsomes from the digestive glands of control and BNF-treated chitons stained with anti-trout LM_2 and LM_{4b}.
Each value represents the mean of three sample replications from the same preparation $\pm$ S.D.

significant increase in the BP metabolizing form LM_{4b} which led to no differences in P-450 content, but significant differences in BPH between BNF-treated and control fish (8).

The large content of P-450 (0.11 nmol/mg) in chiton a primitive herbivore, is consistent with the hypothesis of Nebert and Gonzalez (9) who suggest that cytochrome P-450, already serving a function in steroid biosynthesis early in evolution, broadened its role to include xenobiotic metabolism when animals began to encounter plant metabolites. Since antibodies to the purified trout forms of P-450 crossreacted with the invertebrate protein, there is a strong implication that the enzyme must be structurally conserved through evolution from invertebrates to fish.

It is interesting that anti-LM_2 reacted with two bands, whereas anti-LM_{4b} only recognized one protein. It appears that anti-LM_2 may be more indiscriminate in its epitope identification thus recognizing more sites than anti-LM_{4b}. In mammalian studies, polyclonal antibodies raised against a specific purified P-450 isozyme have been shown to crossreact with other P450s which has led to the use of mRNA quantitation with cDNA probes as a more accurate measure of individual P450 isozymes (9). At any

rate, the two proteins observed in chiton digestive gland samples stained with anti-LM_2 must share some structural similarities.

The measurement of P-450 induction in marine organisms has been suggested to serve as a possible indicator of pollution in marine environments (10). More research needs to be performed focusing on the effect of natural organic chemicals on marine molluscs to determine whether induction of P-450 can occur in nonpolluted environments. These data should allow a better understanding of how marine organisms cope with xenobiotics.

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