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Functional differences in the cytochrome P450 1 family enzymes from Zebrafish (*Danio rerio*) using heterologously expressed proteins

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ABSTRACT

Mammalian cytochrome P450 1 (CYP1) genes are well characterized, but in other vertebrates only the functions of CYP1A genes have been well studied. We determined the catalytic activity of zebrafish CYP1A, CYP1B1, CYP1C1, CYP1C2, and CYP1D1 proteins using 11 fluorometric substrates and benzo[a]-pyrene (BaP). The resorufin-based substrates, 7-ethoxyresorufin, 7-methoxyresorufin, and 7-benzyloxy-resorufin, were well metabolized by all CYP1s except CYP1D1. CYP1A metabolized nearly all substrates tested, although rates for non-resorufin substrates were typically lower than resorufin-based substrates. Zebrafish CYP1s did not metabolize 7-benzyloxyquinoline, 3-[2-(*N*,*N*-diethyl-*N*-methylamino)ethyl]-7-methoxy-4-methylcoumarin or 7-methoxy-4-(aminomethyl)-coumarin. CYP1B1 and CYP1C2 had the highest rates of BaP metabolism. 3-Hydroxy-BaP was a prominent metabolite for all but CYP1D1. CYP1A showed broad specificity and had the highest metabolic rates for nearly all substrates. CYP1C1 and CYP1C2 had similar substrate specificity. CYP1D1 had very low activities for all substrates except BaP, and a different regioselectivity for BaP, suggesting that CYP1D1 function may be different from other CYP1s.

Introduction

Cytochrome P450s (CYPs)[1] are a large superfamily of enzymes that primarily catalyze mixed-function oxidation reactions [1]. CYPs are capable of metabolizing a wide range of substances including steroids [2], pharmaceuticals [3], and xenobiotic compounds [4]. The CYP1 family is important for the metabolism of cyclic structures including polycyclic aromatic hydrocarbons (PAHs) and aromatic amines [5]. The induction of CYP1 catalytic activity, often measured as ethoxyresorufin-*O*-deethylase (EROD) activity, and expression (protein and transcript) have been widely used as a biomarker of exposure and effects to planar halogenated aromatic hydrocarbons in vertebrates.

Vertebrate CYP1 subfamilies include the CYP1A, CYP1B, CYP1C, and CYP1D subfamilies, but not all vertebrate lineages contain all subfamilies or an orthologous CYP1 gene complement. Mammals have three CYP1 genes: CYP1A1, CYP1A2, and CYP1B1. Non-mammalian vertebrates have genes from all the CYP1 subfamilies [6]. Similar to mammals, birds have two CYP1A genes while amphibians and fish have a single CYP1A gene, except for some polyploid species including the salmonids and some frogs [6]. Like their mammalian counterparts, non-mammalian vertebrates have a single CYP1B gene, CYP1B1. The CYP1C subfamily contains the paralogous CYP1C1 and CYP1C2 genes in fish [6]; a single CYP1C gene is thought to be present in birds, amphibians, and reptiles [6]. The CYP1D subfamily has recently been identified in non-mammalian vertebrates, and contains a single CYP1D1 gene [7].

Mammalian CYP1 enzymes are highly inducible by the aryl hydrocarbon receptor [AHR, 8]. CYP1A1 is constitutively expressed at very low levels, but has the highest induction profile by AHR ligands in liver [9]. CYP1A2 and CYP1B1 have substantial hepatic constitutive expression and are induced by AHR ligands, but less then CYP1A1 [5,10]. In zebrafish, CYP1A is the highest constitutively expressed CYP1 in liver [11]. Zebrafish have less constitutively expressed CYP1B1, CYP1C1, CYP1C2, and CYP1D1 in the liver, gill, and kidney compared to CYP1A [7,11]. All zebrafish CYP1s, with the exception of CYP1D1, are upregulated through the AHR [7,11]. Studies of basal expression and induction of CYP1D1 are lacking in other species.

The function of mammalian CYP1s has been well documented, but less is known of other vertebrate CYP1s, outside CYP1A. Mammalian CYP1A1 and CYP1B1 are both able to metabolize PAHs [5] and estrogens [12]; CYP1B1, but not CYP1A1, can metabolize some aromatic amines [5]. CYP1A2 can metabolize estrogens [12] and many aromatic amines [5]. Less overall metabolism is seen from CYP1B1, but the metabolites formed tend to be more highly reactive [5,12,13].

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[1] *Abbreviations used:* AHR, aryl hydrocarbon receptor; BaP, benzo(a)pyrene; CYP, cytochrome P450; PAH, polycyclic aromatic hydrocarbon.

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Previously, we determined estradiol metabolism by zebrafish CYP1s and CYP3A65 [14] in a study that was, to our knowledge, the first to examine functional differences between non-mammalian vertebrate CYP1s. Zebrafish CYP1A and CYP1B1 metabolized estradiol with similar regioselectively to their mammalian orthologs [14]. Zebrafish CYP1A, CYP1C1, and CYP1C2 metabolized estradiol with a similar regioselectivity, but CYP1C2 metabolized estradiol at a lower rate than CYP1A and CYP1C1 [14]. Heterologous expression of the CYP1s was optimized for our research using the proto-typical CYP1 substrate 7-ethoxyresorufin (ER). Zebrafish CYP1A, CYP1B1, CYP1C1, and CYP1C2 were all able to metabolize ER [14]. CYP1D1 did not metabolize estradiol or ER at a high catalytic rate [14].

Catalytic activity and substrate specificity of individual CYP isoforms can be determined [13,15] using synthetic substrates that produce fluorescent metabolites [16] or analytical detection of metabolites produced from model compounds. For CYP1s, model compounds such as BaP [17] and fluorescent assays based on the alkoxyresorufin compounds, including ER, are appropriate as they are known mammalian CYP1 substrates [18]. As the function of the non-mammalian CYP1 genes CYP1C1, CYP1C2, and CYP1D1, are not clear, inclusion of atypical mammalian CYP1 substrates may be appropriate to discern novel function not seen in mammalian species where these genes are lacking. Here, we present the first detailed catalytic assessment of the substrate specificity of CYP1s from a non-mammalian vertebrate. Zebrafish CYP1A, CYP1B1, CYP1C1, CYP1C2, and CYP1D1 have previously been cloned and co-expressed with human CYP reductase [14]. We have tested the metabolic capabilities of CYP1s using BaP and 11 synthetic fluorescent-based CYP substrates. The fluorescent substrates are comprised of four resorufin based compounds, five coumarin compounds, a fluorescein substrate, and a quinoline. This data will help to determine the functional roles of non-mammalian CYP1s and suggest whether the novel CYP1Cs and CYP1D proteins have overlapping or novel functions.

Methods

Cloning, expression, and purification of zebrafish CYPs

Zebrafish CYP1A, CYP1B1, CYP1C1, CYP1C2, and CYP1D1 were cloned and co-expressed with human cytochrome P450 reductase in *Escherichia coli* JM109 cells, and purified bacterial membrane fractions were isolated as previously reported [14]. Bacterial expression utilized a strategy for expression without 5′ modification [19]; each CYP gene was cloned with the *ompA*2+ leader sequence to allow targeting of the protein to the bacterial outer membrane. This sequence is excised after insertion of the protein into the membrane allowing expression of full-length vertebrate membrane proteins in bacteria [19]. Expression was optimized for each cloned CYP with the addition of 0–1 mM δ-aminolevulinic acid (Ala; MP Biomedicals, Solon, OH) and functional proteins confirmed with CO-difference spectra and catalytic assays. Optimal Ala concentrations for each insert were determined to be 0.1 mM for all CYP constructs except CYP1A and CYP1B1 where Ala was added at 0.5 and 1.0 mM, respectively [14].

Fluorescent based catalytic assays

All substrates and metabolites analyzed, as well as fluorescent wavelengths and concentrations used are shown in Table 1. Reactions involving the resorufin-based substrates 7-ethoxyresorufin (ER), 7-methoxyresorufin (MR), 7-pentoxyresorufin (PR), and 7-benzyloxyresorufin (BR) were in 50 mM Tris, 0.1 M NaCl, pH 7.8. Reactions involving 7-benzyloxy-4-(trifluoromethyl)coumarin (BFC), 7-methoxy-trifluoromethylcoumarin (MFC), 7-benzyloxyquinoline (BQ), dibenzylfluorescein (DBF), 3-cyano-7-ethoxycoumarin (CEC), 3-[2-(*N*,*N*-diethyl-*N*-methylamino)ethyl]-7-methoxy-4-methylcoumarin (AMMC), and 7-methoxy-4-(aminomethyl)-coumarin (MAMC) were in 0.5 M potassium phosphate buffer, pH 7.5. Reactions were initiated with 1.33 nM NADPH (Sigma, St. Louis, MO) and all were assayed at 30 °C. Assays were run at 30 °C because this temperature represents a physiologically relevant temperature for this species; most zebrafish are maintained in laboratories at 28–30 °C. Catalytic rates do not appear to be significantly altered when assay temperature is 30 ± 5–10 °C (Scornaienchi and Wilson, unpublished data). All resorufin-based substrates (ER, MR, PR, BR) were from Sigma (St. Louis, MO) and BFC, MFC, BQ, DFB, CEC, AMMC, and MAMC were from BD Gentest (Woburn, MA). Catalytic activity was determined using a kinetic assay and normalized for total P450 content. The fluorescent assays have been optimized for fish in our laboratory using rainbow trout and killifish liver microsomes [20] with additional temperature optimization for zebrafish proteins (as described above).

Benzo[a]pyrene metabolism

Incubations contained 0.1 M sodium phosphate, pH 7.4; 0.25 mg ml^{-1} human microsomal epoxide hydrolase (BD Biosciences, San Jose, CA); 1 mM NADPH, membrane fractions containing 100 mg total protein of recombinant zebrafish CYPs co-expressed with human CYP reductase; and benzo[a]pyrene (BaP; Accustandard, New Haven, CT) at a final concentration of 0, 20, 40, 80, or 120 μM. BaP was dissolved in DMSO such that the final concentra-

Table 1
Fluorescent cytochrome P450 assay conditions. The concentrations used and metabolites detected are given for each substrate. Substrate abbreviations are shown in brackets. The excitation (Ex) and emission (Em) conditions are shown as the wavelength/bandwidth of filters used and the wavelength was always within 10 nm of the optimal wavelength for each metabolite.

Substrate	Concentration (μM)	Metabolite	Ex (nm)	Em (nm)	Human CYP specificity
7-Ethoxyresorufin (ER)	2	Resorufin	540/35	590/20	1A1 > 1A2 = 1B1
7-Methoxyresorufin (MR)	5	Resorufin	540/35	590/20	1A2
7-Benzyloxyresorufin (BR)	5	Resorufin	540/35	590/20	1A, 2B, 3A
7-Pentoxyresorufin (PR)	5	Resorufin	540/35	590/20	2B
7-Benzyloxy-4-(trifluoromethyl)coumarin (BFC)	1000	7-Hydroxy-4-(trifluoromethyl)coumarin (HFC)	400/30	528/20	3A4
7-Methoxy-trifluoromethylcoumarin (MFC)	1000	7-Hydroxy-4-(trifluoromethyl)coumarin (HFC)	400/30	528/20	2C9, 2C19, 1A2
7-Benzyloxyquinoline (BQ)	1000	7-Hydroxyquinoline (HQ)	400/30	528/20	3A4
Dibenzylfluorescein (DBF)	10	Fluorescein	485/20	528/20	2C8, 2C9, 3A4
3-Cyano-7-ethoxycoumarin (CEC)	10	3-Cyano-7-hydroxycoumarin (CHC)	400/30	460/40	2C9, 2C19, 1A2
3-[2-(*N*,*N*-Diethyl-*N*-methylamino)ethyl]-7-methoxy-4-methylcoumarin (AMMC)	50	3-[2-(*N*,*N*-Diethylamino)ethyl]-7-hydroxy-4-methylcoumarin hydrochloride (AHMC)	400/30	460/40	2D6
7-Methoxy-4-(aminomethyl)-coumarin (MAMC)	50	7-Hydroxy-4-(aminomethyl)-coumarin (HAMC)	400/30	460/40	2D6