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Structural features of cytochrome P450 1A associated with the absence of EROD activity in liver of the of the loricariid catfish *Pterygoplichthys* sp

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Abstract

The Amazon catfish genus *Pterygoplichthys* (Loricariidae, Siluriformes) is closely related to the loricariid genus *Hypostomus*, in which at least two species lack detectable ethoxyresorufin-*O*-deethylase (EROD) activity, typically catalyzed by cytochrome P450 1 (CYP1) enzymes. *Pterygoplichthys* sp. liver microsomes also lacked EROD, as well as activity with other substituted resorufins, but aryl hydrocarbon receptor agonists induced hepatic CYP1A mRNA and protein suggesting structural/functional differences in *Pterygoplichthys* CYP1s from those in other vertebrates. Comparing the sequences of CYP1As of *Pterygoplichthys* sp. and of two phylogenetically-related siluriform species that do catalyze EROD (*Ancistrus* sp., Loricariidae and *Corydoras* sp., Callichthyidae) showed that these three proteins share amino acids at 17 positions that are not shared by any fish in a set of 24 other species. *Pterygoplichthys* and *Ancistrus* (the loricariids) have an additional 22 amino acid substitutions in common that are not shared by *Corydoras* or by other fish species. *Pterygoplichthys* has six exclusive amino acid substitutions. Molecular docking and dynamics simulations indicate that *Pterygoplichthys* CYP1A has a weak affinity for ER, which binds infrequently in a productive orientation, and in a less stable conformation than in CYP1As of species that catalyze EROD. ER also binds with the carbonyl moiety proximal to the heme iron. *Pterygoplichthys* CYP1A has amino acids substitutions that reduce the frequency of correctly oriented ER in the AS preventing the detection of EROD activity. The results indicate that loricariid CYP1As may have a peculiar substrate selectivity that differs from CYP1As of most vertebrates.

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Keywords

CYP1A; Ethoxyresorufin; Substrate specificity; Amino acid; Biotransformation

1. Introduction

Cytochrome P450 family 1 (*CYP1*) genes code for biotransformation enzymes in all vertebrate groups [1, 2]. CYP1As in fish and CYP1A1 in mammals are strongly induced by and metabolize aryl hydrocarbon receptor (AHR) agonists (e.g. planar halogenated and aromatic hydrocarbons). These enzymes also are implicated in the toxic effects of many of such compounds [3]. Ethoxyresorufin-*O*-deethylase activity (EROD) is widely used as a marker of CYP1A activity and induction [4–6]. Other CYP1 proteins (CYP1A2 and CYP1B1 in mammals and CYP1B1, CYP1C1, CYP1C2, and CYP1D1 in fish) also can catalyze EROD [7–9], but function of expressed proteins and immunoassays indicate that CYP1A is the most active catalyst for this activity in fish liver [10, 11].

Recently, we reported that we could not detect EROD activity in microsomal preparations of liver and other organs from two fish species in the genus *Hypostomus* family Loricariidae (Siluriformes), either from control fish or fish treated with potent AHR agonists [12]. The lack of EROD activity suggests that CYP1s, and perhaps especially the CYP1As in the loricariids may differ in structure and function from those of other vertebrates. The CYP1A amino acid sequence is remarkably conserved among vertebrates, with a common three-dimensional structure and similar substrate affinities and functions [1]. However, single non-synonymous amino acid substitutions are able to dramatically change CYP1A1 and CYP1A2 specificity in site-directed mutagenesis studies with heterologously expressed mammalian protein, which may alter xenobiotic detoxification, activation of pro-mutagens, and cancer susceptibility [13–16].

In this study, we examined two other members of the Loricariidae family, *Pterygoplichthys* sp. and *Ancistrus* sp., and also *Corydoras* sp. (Callichthyidae), a siluriform from a different family, to assess whether other Siluriformes also lack EROD activity and to provide a structural understanding for this unusual phenotype. Similar to *Hypostomus*, *Pterygoplichthys* lacks microsomal EROD activity, as well as activity with other substituted resorufins (methoxy-, pentoxy- and benzyloxyresorufin). The genus *Pterygoplichthys* is more closely related to *Hypostomus* than is *Ancistrus* while *Corydoras* more distantly related (Supplemental Figure 1). CYP1As were cloned from complementary DNA (cDNA) and sequenced, and CYP1A transcript, protein, and enzyme activities were assessed in liver of fish exposed to the AHR agonists β -naphthoflavone (BNF) or 3,3',4,4',5-pentachlorinated biphenyl (PCB126).

To further understand the nature of this rare condition, we sought a structural basis for the lack of ER metabolism by generating homology models for the *Pterygoplichthys* CYP1A and performing docking studies followed by molecular dynamics simulations for ER binding *in silico*. All of these procedures were extended to the CYP1A of the phylogenetically related species (*Ancistrus* sp.) that is able to catalyze EROD, and to CYP1A of zebrafish (*Danio rerio*; Cypriniformes; Cyprinidae), which is not closely related to the Siluriformes. The results suggest unusual active site features that could produce the anomalous substrate specificities of CYP1A of some loricariid species.

2. Material and methods

2.1 Fish handling and exposure

Species in three genera of Siluriformes fishes were used in this study; two from the Loricariidae family (*Pterygoplichthys* and *Ancistrus*) and one from the Callichthyidae (*Corydoras*). All fishes were purchased from a local supplier in Falmouth, MA USA. *Pterygoplichthys* identity was further characterized by the PCR amplification and sequencing of internal transcribed spacer (ITS) and partial tRNA-Pro/D-loop/tRNA-Phe conserved fragments from genomic DNA [17]. Sequences (Supplemental File 2) were blasted against GenBank showing that the species used in this study was most closely related to *Pterygoplichthys scrophus* (synonyms, *Liposarcus scrophus* and *Glyptoperichthys scrophus*).

Fishes were acclimated in aquaria in the laboratory for one week in re-circulating filtered water at 28°C. Fish were intraperitoneally (i.p.) injected with 50 mg/kg of β -naphthoflavone (BNF) or 50 mg/kg of 3,4,3',4',5' pentachlorobiphenyl (PCB126) in DMSO, or to DMSO [12]. A second batch of *Pterygoplichthys* and zebrafish (*Danio rerio*) were exposed to 100 μ M PCB126 added in DMSO in static water exposures for 24 hours, followed by 48 hours in re-circulating charcoal filtered water [18, 19]. Fish were sampled three days after initiation of exposure. A total of 31 *Pterygoplichthys*, 7 *Ancistrus*, 11 *Corydoras* and 30 Zebrafish were used in this study. The number of fish in each experimental group is described on the legend of figure 1.

2.2 EROD determination and CYP1A protein detection

Hepatic microsomes were prepared as described elsewhere [20] for spectrofluorimetric EROD determination [20] and CYP1A detection by immunoblotting [21]. For *Corydoras* and zebrafish, livers from all fish of each group were pooled for microsome preparation due to the small fish size. For the other two species, microsomes were prepared from individual livers. EROD reactions in microsomes were started by the addition of NADPH to reaction mixture and fluorescence accumulation was determined using a Cytofluor at 30°C. EROD values were normalized to total microsomal protein content [22] and expressed as pmol of resorufin minute⁻¹ milligram of protein⁻¹. CYP1A protein was detected in liver microsomes by western blotting using a monoclonal anti-scup CYP1A antibody (MAb1-12-3) [23]. Secondary antibodies were coupled with a fluorophore (IRDye 800, LI-COR Biosciences, San Diego, CA) and signal was captured in the Odyssey Infrared fluorescent dual laser scanner (LI-COR) using laser excitation at 800nm (sample detection) and 700nm (BioRad Precision Plus™ blue prestained protein standard detection).

NADPH consumption by liver microsomes was measured using a Shimadzu dual beam spectrophotometer. Liver microsomes were diluted in buffer to 0.3 ml in 50 mM TRIS, 100 mM NaCl, pH 7.8 buffer with 0.106 μ M NADPH and divided between sample and reference cuvettes (0.26 μ M/ml final). Baseline was determined and then 50 μ l of buffer was added to the reference cuvette and 50 μ l buffer + 8 μ M ER (2 μ M final) was added to the sample cuvette. The time course of loss of absorbance at 340 nm was recorded for 4 minutes, then any change in absorbance at 572 nm was recorded for 3 minutes. Calculations were made using appropriate extinction coefficients from linear portions of absorbance vs time plots.

2.3 CYP1A transcript quantification and cloning

Samples of individual livers were collected for RNA extraction using STAT-60 (Invitrogen) followed by DNase treatment. RNA quantity and quality was determined spectrophotometrically (Nanodrop ND1000, NanoDrop Technologies, Wilmington, DE).

cDNA was prepared following the manufacturer's instructions for the Omniscript Reverse Transcriptase (Qiagen, Inc., Valencia, CA) with anchored oligo(dT) primers (MWG Biotech, Inc., High Point, NC) and RNasin RNase inhibitor (Promega Corp. Madison, WI). Quantitative real time PCR (qPCR) reactions were carried out using iQ SYBERGreen Supermix and an iQ Real-Time PCR Detection System (Bio-Rad) with gene-specific CYP1A and β -actin (normalizing control) primers (Supplemental material table 1). Gene expression was analyzed in triplicate using the following cycling parameters: 95°C for 3 minutes and 40 cycles of 95°C for 15s and 62°C for 25s. Primers specificity was determined by the melt curve at the end of each PCR run and by cloning and sequencing the amplicon. Modifications in gene expression were calculated in relation to expression levels in each control group and normalized against β -actin expression by the $\Delta\Delta C_t$ method [24, 25].

To sequence the full length CYP1A cDNA in the three genera, primers sets were designed to regions of the CYP1A gene conserved between *Peltobagrus fulvidraco* (a siluriform) and a number of other fishes species, followed by RACE reactions to amplify the 5' and 3' ends (BD SMART™ RACE, Clontech). Amplicons from two independent PCR reactions from at least five individuals from each species were gel purified and cloned into pGEM-t Easy Vector (Promega), which subsequently were used to transform *E. coli* (TOP10 kit, Invitrogen). Plasmids from at least three bacteria colonies were purified (QiaPrep™, Qiagen) and sequenced (MWG biotech). All primers information can be found in supplemental material.

2.4 CYP1A modeling, ethoxyresorufin docking and molecular dynamics

CYP1A homology models were built based on the human CYP1A2 crystal structure (PDB: 2HI4) [26]. The N-terminal membrane anchor regions of the fish CYP1A sequences were truncated to match the sequence of the human structure. Fish CYP1A amino acid identities to the human template ranged from 53–55%. Sequences were initially aligned using ClustalW and refined using the *salin_2d* function of Modeller 9.5 [27–30]. Default parameters in Modeller were applied, excluding water molecules and any ions that were part of any of the templates with the exception of the heme and heme iron. Homology modeling was carried out by satisfaction of spatial restraints using the automodel function of Modeller, with very thorough VTFM, thorough MD, and two repeat cycles of minimization. Ten randomly seeded models were generated for each protein. Both Procheck [31] and the Modeller DOPE Score were used to assess model quality and pick one best homology model for further work.

Molecular docking and dynamics were performed essentially as in Da Silva et al., [32]. Briefly, the ethoxyresorufin structure, topology and other relevant files were generated with Dundee PRODRG server [33]. Rigid receptor/flexible ligand docking and docking energy calculations were performed with AutoDockTools and AutoDock 4.0 using the Lamarckian genetic algorithm [34]. The 100 lowest energy conformations were selected by the genetic algorithm after 27,000 generations. To fit the entire protein the grid size was 126x126x126 ÅÅ with 0.375 ÅÅ between any grid points in all simulations. Docked conformations were analyzed following the criteria established by Tu et al [35], in which for an effective binding the distance between the hydrogen to be abstracted and the ferryl oxygen must be less the 3.5 ÅÅ and the angle among those atoms and the carbon at the oxidation site must be less than 120°. The most frequent docked conformation and the conformation that best matched these criteria were subjected to 10 ns of molecular dynamics simulation using the program GROMACS applying the GROMOS96 53a6 force field at 300K without any restrictions [36]. Standard water solvation was performed, using a cubic water box of approximately 110x110x110 ÅÅ with periodic boundaries. Before the MD run the system was minimized by the steepest descent and a conjugate gradient followed by a 500 ps step of protein solvation.

3. Results

3.1 Induction of CYP1A and activity

EROD activity—EROD activity was not detected in control *Pterygoplichthys*, or in *Pterygoplichthys* exposed to the AHR agonist PCB126, either intraperitoneally (i.p.) or in the water (Figure 1A). In contrast, in *Ancistrus* and *Corydoras*, EROD activity was detected with liver microsomes of all control fish, and activity was induced in fish treated i.p. with PCB126. As expected, EROD was detected in control and PCB126-exposed zebrafish, used as a positive control, *Ancistrus*, the other loricariid, had very low activity, less than $2 \text{ pmol min}^{-1} \text{ mg}^{-1}$ in control and $42 \text{ pmol min}^{-1} \text{ mg}^{-1}$ in PCB-treated fish. In contrast, *Corydoras* EROD activity was induced more than 50-fold by PCB, to $135 \text{ pmol min}^{-1} \text{ mg}^{-1}$ (Figure 1A). EROD activity also was not detected in *Pterygoplichthys* exposed i.p. to BNF, while in *Ancistrus* and *Corydoras*, EROD was induced by BNF, with responses similar to those obtained with PCB126 (data not shown). All *Pterygoplichthys* individuals exhibited the same phenotype (no EROD detection), which suggests that this phenotype reflects the dominant genotype rather than a polymorphism within the species. *Pterygoplichthys* liver microsomes also were assayed for activity with other resorufin substrates (methoxy-, pentoxy and benzyloxyresorufin), but no activity was detected with any of these substrates, with microsomes from either control or AHR-agonist exposed fish.

The possibility that some component of *Pterygoplichthys* liver microsomes directly inhibits EROD or quenches resorufin fluorescence was examined by analyzing EROD activity in microsomes prepared from livers of known high activity BNF-treated scup (*Stenotomus chrysops*) [37] mixed with livers from *Pterygoplichthys*. The mixed tissue microsomes yielded activity equivalent to the amount of scup liver in the mixture indicating that no inhibitory component was present in the *Pterygoplichthys* liver.

CYP1A mRNA expression—The levels of CYP1A transcript in the three siluriform species and zebrafish were assessed using qPCR. PCB126 i.p. induced transcript expression in all four species, and the induction profile in the three Siluriformes followed the same trend as observed for EROD (Figure 1B). BNF also induced transcript in these species, although in contrast to PCB126 effects, the induction by the BNF treatment was greater in *Pterygoplichthys*, than in *Ancistrus* or in *Corydoras* (data not shown). Waterborne PCB126 also induced CYP1A transcript expression in both *Pterygoplichthys* and zebrafish (Figure 1B).

CYP1A protein expression—Immunoblot assay of microsomal proteins with monoclonal antibody 1-12-3 to scup CYP1A, which cross-reacts with CYP1A in other vertebrates [11], detected a single immunoreactive band in both control and AHR agonist-exposed *Pterygoplichthys* (Figure 2), as well as in the other three species. Basal antigenic signal levels in control fish were similar among the siluriform species ($\text{OD} = 1.0 \pm 0.2$) and higher in zebrafish ($\text{OD} = 9.4 \pm 1.9$). With i.p. PCB126 treatment, the CYP1A signal increased relative to controls in *Ancistrus* ($\text{OD} = 3.0 \pm 0.1$) and *Corydoras* ($\text{OD} = 7.5$) but changed little in *Pterygoplichthys* ($\text{OD} = 0.9 \pm 0.2$) (Figure 1C). Waterborne PCB126 elicited a modest increase in *Pterygoplichthys* CYP1A ($\text{OD} = 1.8 \pm 0.4$) but a much larger increase in CYP1A content in *Danio* ($\text{OD} = 70.7 \pm 11.6$) (Figure 1C). BNF treatment caused a similar induction of CYP1A protein in *Pterygoplichthys* ($\text{OD} = 3.3 \pm 0.6$) and *Ancistrus* ($\text{OD} = 3.5$) and a larger increase in *Corydoras* ($\text{OD} = 6.7$).

3.2 CYP1A sequence

Cloning and sequencing of the *Pterygoplichthys* CYP1A gave a full-length sequence (GI: 312982586) coding for a 521 amino acid protein with 75% identity to its zebrafish

counterpart (GI: 40538769), 58% identity to the *Xenopus* CYP1A1 (GI: 147901868), 57% identity to the chicken CYP1A4 (GI: 45384061), 56% identity to the cat CYP1A1 (GI: 67972629), 58% identity to the rat CYP1A1 (GI: 46048640), 57% identity to the Human CYP1A1 (GI: 189339226). The *Ancistrus* (GI: 312982588) and *Corydoras* (GI: 312982590) sequences translate to 521 and 515 amino acids, with 77% and 75% identity to zebrafish, respectively.

Comparing the translated CYP1A sequences from *Pterygoplichthys*, *Ancistrus*, and *Corydoras* to CYP1As from a set of 24 other fish species, it was found that these three siluriform species share 17 substituted positions that are not shared by any of the other species. Of those 17 modifications, nine modifications changed the amino acid physicochemical properties (non-conservative replacement) relative to the residues in the 24 other species, and three are located inside or within two residues from the boundary of a putative substrate recognition site (SRS) [38].

The Loricariidae fish (*Pterygoplichthys* and *Ancistrus*) also have 22 amino acid substitutions in common but that are not shared by *Corydoras*. Eight of these are nonconservative and five are located at or adjacent to SRSs. Six amino acid substitutions were exclusive to *Pterygoplichthys*: four at positions 32, 59, 94 and 159, that are nonconservative, one at position 255, adjacent to SRS3, and another at position 98, that is neither non-conservative nor close to an SRS. Among all of the 48 substitutions found in the three siluriforms, twelve are located in positions that are absolutely conserved in the other 24 species. At these positions, the same amino acid occurred in the three siluriform species four times, and six times in the two Loricariidae. The glycine at position 32 was found exclusively in *Pterygoplichthys*; arginine is found at that position in all of the other species examined to date. These results are summarized in Table 1 and the supplemental material.

3.3 CYP1A modeling, ethoxyresorufin docking and molecular dynamics for *Pterygoplichthys*, *Ancistrus*, and Zebrafish CYP1As

The sequence data were used to construct three-dimensional (3D) models of the inferred CYP1A proteins of *Pterygoplichthys* and *Ancistrus*, which were used for substrate docking and molecular dynamics analyses. Models were based on the human CYP1A2 crystal structure as a template (PDB:2HI4) [26] and were deposited in the Protein Model Data Bank (PMDB IDs: PM0077253 and PM0077252). The overall conformation of the models of the two CYP1As was extremely similar (root mean square (RMS) 1.1–2.2 Å for all N, C, C α , O), with the exception of the distance between the heme iron and the conserved cysteine ligand for the heme. In *Pterygoplichthys*, this distance was 3.0 Å while in *Ancistrus* and *Danio* (PMDB IDs: PM0077255) the distance was shorter (2.1 Å) and more similar to that in human CYP1A2 crystal structure (2.3 Å). This difference appears to be due to a more planar heme Fe conformation in the *Pterygoplichthys* model relative to the out-of-plane positioning of the Fe in the CYP1A2 crystal structure.

Rigid receptor docking simulations for *Pterygoplichthys*, *Ancistrus* and *Danio* CYP1A models were performed using AutoDock. Docking positions were determined for 100 minimum energy poses following a thorough Lamarckian genetic algorithm search. Two metrics were examined: the proportion of lowest energy poses within the active site, and the proportion of docking attempts displaying the correct orientation of ER for deethylation.

Docking of ER to *Ancistrus* CYP1A exhibited the highest frequency of ligand poses inside the active site (69%), while the *Danio* CYP1A model had only 37% of the ligand poses inside the active site (Figure 3). Only 17% of the total minimum energy poses displayed ER docked in the active site of the *Pterygoplichthys* CYP1A model. The majority of the docking

replicates had the ER substrate docked at two pockets outside of the active site (59% and 14%) (Figure 3).

Far fewer low energy poses displayed ER docked in the active site in the correct orientation for deethylation to occur (with the ethoxy group oriented toward the heme). For *Ancistrus* and *Danio*, 22% and 15% of the total poses were in a productive orientation, respectively. In contrast, in *Pterygoplichthys* CYP1A merely 5% of the total number of poses were in a putatively productive conformation for deethylation. The other poses in the *Pterygoplichthys* CYP1A active site showed ER docked with the ethoxy group away from the heme, and the ring with the carbonyl positioned toward the heme iron, at a distance of 4.5 Å (Figure 4). There were ER poses with this orientation also in *Ancistrus* CYP1A, although not as close to the Fe as in *Pterygoplichthys*. No poses with ER in this orientation were found in the zebrafish CYP1A.

Ten nanosecond molecular dynamic simulations were run for *Pterygoplichthys*, *Ancistrus*, and *Danio* CYP1As, starting from the conformation with the shortest distance between the ethoxyresorufin methylene carbon of the ethoxy group and the iron atom of the heme group. At the end of the simulation, the three systems were energetically stable (Supplemental figure 2). The backbone root-mean-squared deviation (RMSD), which is the overall distance each atom traveled during the simulation and a measure of conformational stability, showed that only *Pterygoplichthys* CYP1A did not reached a stable plateau (Figure 5B). The backbone root-mean-squared fluctuation (RMSF), which is the flexibility of each amino acid during the simulation, revealed two common flexible areas present in all the three CYP1A models; the first extending from the amino acid residue 293H to residue 300D, other from residue 439S to 446N and only in *Pterygoplichthys* model a third flexible area, from 146A to 161S (Figure 5C). Moreover, the distance between the heme iron and the ER methylene carbon tended to increase over time only in the *Pterygoplichthys* system (Figure 5A) while in *Danio* and *Ancistrus*, it remained stable for most the simulation.

The molecular dynamics showed that in *Pterygoplichthys* CYP1A the distance between ER and the heme iron tended to increase for otherwise productive orientations. However, the docking results also showed that poses with the carbonyl end of ER positioned toward the heme iron were favored, albeit infrequently. This suggests the possibility of oxidation at this part of the ethoxyresorufin molecule. To further examine this hypothesis we tested used liver microsomes from BaP-treated and control *Pterygoplichthys* for NADPH consumption under ethoxyresorufin, which occurred at a excessive rate ($1 \text{ nmol min}^{-1} \text{ mg}^{-1}$ of microsomal protein). These same liver microsomes showed no formation of resorufin.

4. Discussion

The catalytic capability of CYP1 enzymes is important to the susceptibility of organisms to a variety of toxic chemicals [5]. Substituted resorufins are generally accepted as reliable marker substrates for vertebrate CYP1s, especially CYP1As. In the present study, we have shown that *Pterygoplichthys* sp., a loriciid fish, is unable to catalyze the deethylation of ethoxyresorufin. EROD activity was detected in *Ancistrus* sp., also a member of the Loricariidae family, and in *Corydoras* sp., a member of the Callichthyidae family, although belonging to the same order (Siluriformes) as the Loricariidae. A complete absence of EROD detection in liver microsomes from wild-type vertebrates has been reported previously only in *Hypostomus* species (*H. affinis* and *H. aurobutatus*) [12], and in the primitive agnathan lamprey *Petromyzon marinus* [39, 40]. Thus, among the Gnathostomata, *Pterygoplichthys* and *Hypostomus* spp. so far appear to be unique in lacking detectable EROD activity.

It is perhaps significant that *Pterygoplichthys* and *Hypostomus* are very closely related phylogenetically, and in fact *Pterygoplichthys* previously had been classified in the genus *Hypostomus* (See references [41, 42] for recent systematic studies of the Siluriformes order). *Ancistrus* is more closely related to both *Pterygoplichthys* and *Hypostomus* than is *Corydoras*, which is classified in a different family (Supplemental Figure 1). While in *Pterygoplichthys* and *Hypostomus* the lack of EROD is complete, in *Ancistrus* basal EROD activity is barely above the detection limit and, in this study, *Corydoras* presents the highest EROD values in both control and exposed siluriform species, indicating that EROD might follow a phylogenetic trend. Several other well-known Siluriformes (catfishes) including the African catfish, *Clarias gariepinus*, and the North American brown bullhead, *Ameiurus nebulosus*, also exhibit relatively low EROD activity either in control or in AHR-agonist exposed animals [43–46]. Thus, CYP1 evolution in the Siluriformes order appears to have resulted in a decline of the capacity to catalyze EROD activity, with absence in some members of the Loricariidae family. It is possible that the CYP1A expression detected is a “vestigial” up-regulation of an ancient response, of what is becoming a pseudogene in the loricariid line. However, we suggest that there are likely to be other functions of this CYP1A and current studies are addressing this question with heterologously-expressed enzyme. In other fish there are other CYP1s that are inducible by AHR agonists and that can catalyze EROD [10]. If homologues occur in the loricariids, the lack of microsomal EROD suggests that they would not be expressed at meaningful levels in liver, or that they too are deficient in EROD activity.

Given the prominence of EROD as a diagnostic and biomarker CYP1A substrate, we focused our efforts to understand the lack of EROD activity on this CYP1 enzyme. All three novel species used in this study were shown to possess a *CYP1A* gene and to express CYP1A mRNA and protein, which excludes loss of the gene as an explanation for the lack of activity. The CYP1A gene not only is present in those fishes, but its expression is induced by AHR agonists. All three siluriform species we examined here, showed mRNA induction in a range of response comparable to the degree of expression observed in *Danio rerio* (20–150 fold), (Figure 1B) indicating that they have intact CYP1A induction mediated through the AHR receptor mechanism.

The capacity of *Pterygoplichthys* to accumulate CYP1A protein differs from *Danio* and many other fish species, which under similar exposure conditions exhibit higher levels of CYP1A protein induction after exposure to AHR agonists [47–49]. We found at most a 2-fold increase in *Pterygoplichthys* CYP1A protein, as compared to a 7.5-fold increase in zebrafish under the same treatment conditions. A relatively weak induction of CYP1A protein in response to potent AHR agonists has been reported in other siluriform species, including *Ictalurus punctatus*, *Ameiurus nebulosus* and *Clarias gariepinus* [43, 44, 46, 50–52], although strong induction of CYP1A protein has been observed in liver and kidney of *Ameiurus nebulosus* collected from a U.S. Superfund site (unpublished observations). Regardless, our data indicate that in some loricariids, liver microsomes bearing CYP1A protein have little or no capacity for EROD, implying that CYP1A structural features could be responsible for this phenotype.

In total, 48 amino acid positions were substituted in *Pterygoplichthys*, *Ancistrus* and *Corydoras* as compared to CYP1As from a set of 24 other fish species. It is possible that those substitutions, or a sub-set of them, decrease the affinity of *Ancistrus* and *Corydoras* CYP1A protein toward ER, evident as a relatively low basal EROD activity. Remember that only one substitution can lead to a defective CYP1A [13–16]. The six changes specific to *Pterygoplichthys*, together with the 42 changes which are shared with *Ancistrus* and *Corydoras*, could reorganize the 3D protein structure in a way that ER is stabilized outside the active pocket and unstable inside of it, as predicted by molecular dynamics, leading to

undetectable levels of EROD activity as shown experimentally. However, due to the large number of shared substitutions, those predictions could not be tested by *in silico* amino acid changes followed by ER docking and no unusual feature was observed inside the active site. We are currently sequencing *CYP1A* genes in a greater number of Loricariidae species in an attempt to identify substitutions to test *in silico* and reverse *in vitro* by site directed mutagenesis and heterologous expression to determine those changes critical to the observed phenotype of no EROD activity in these fishes. It has been shown by site directed mutagenesis that single amino acid substitutions are able to abolish EROD activity in heterologously-expressed human CYP1A1 [14–16, 35, 53].

Docking studies with well established P450 substrates often results in alternative poses together with the most effective pose for a particular activity. In *Pterygoplichthys*, these alternative poses, however, were the most frequent and the frequency of the most effective pose for EROD was low. It was thought that, although not capable of catalyzing the EROD reaction, *Pterygoplichthys* CYP1A might be catalytically active in oxidizing ER, but not at the ester bond. This hypothesis was strengthened by the detection of NADPH consumption above the endogenous level (background) when ER was added to microsomal preparations. Our docking results on the orientation of ER in the active site suggest that oxidation could occur at the opposite end of ER, at the C2 carbon.

As shown by the dockings, ER binds appropriately for oxidation but not in the most usual and effective pose for EROD. As no evident structural feature was found to prohibit ER to dock in the AS in the most usual conformation, we have calculated CYP1A flexibility, a property that enables substrate movement into the protein core. The first common flexible area (residues 293H – 300D) is located at the end of H-helix and forms most of the H-I loop, in close proximity to the putative main substrate access channel. The reduced flexibility of this area in *Pterygoplichthys* CYP1A model in comparison to the models of the other species might narrow this major substrate entrance and influence enzyme selectivity. The second common flexible area (439S – 446N) is located at the K' loop, close to the end of the I-helix. The flexible area that was found solely in *Pterygoplichthys* (146A – 161S) is located at the end of C-helix and forms most of the C-D loop, close to the first flexible area and to the heme. Despite the relatively few poses of correctly oriented ER inside the *Pterygoplichthys* CYP1A active site, the molecular dynamics also showed that those poses are not as stable as observed for the *Ancistrus* and *Danio* CYP1A active sites. Together our results suggest that other amino acids in addition to those located inside the putative CYP1A active site and SRSs play important roles in the enzyme specificity and regioselectivity. Changes outside the SRSs might interfere with those enzyme properties by altering the conformation of access channels and/or the protein flexibility, leading to oxidation at unconventional molecule regions.

Ethoxyresorufin and the other substituted resorufins are not biologically or environmentally relevant chemicals. To what extent the amino acid substitutions observed in *Pterigoplythcthyis* CYP1A might change the affinity and regioselectivity of this enzyme for environmentally relevant substrates is unknown. We have shown that *Hypostomus* individuals are able to convert DMBA into an active mutagen, generating a high frequency of micronucleated erythrocytes [12]. Whether this is due to the action of CYP1A or CYP1B1 or some other CYP1 on DMBA is not known. The siluriform *Ameiurus nebulosus* is more sensitive to chemical carcinogenesis than *Ictalurus punctatus* (channel catfish), a close relative that displays both higher EROD basal values and induction [43, 46]. Determining the sequence and capacity of other loricariid CYP1s for ER, as well as their activity toward pro-carcinogens, will be important to understanding the environmental relevance of this adaptation.

The biological significance of unusual catalytic capabilities of *Pterigoplythchthys* CYP1A suggested by the absence of EROD activity is unknown. Further analysis of the CYP1 family in Loricariidae species promises a better understanding of the processes governing vertebrate CYP1 functional evolution, as well as their involvement with chemical carcinogenesis and species-environment interactions. The loricariid species offer an opportunity to investigate environmentally or physiologically relevant substrates for CYP1A, and to assess whether the metabolism of natural substrates correlates directly with EROD or not, which would give greater weight to the use of this activity as a biomarker of exposure to AHR agonists. Modeling and docking studies will provide an essential component of such studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. CYP1A activity - EROD (A), CYP1A transcript (B) and CYP1A protein (C) fold inductions by intraperitoneal exposure (black bars) and exposure through the water (white bars) to PCB126 in

Pterygoplichthys (Ptery, n = 2 and 8), *Ancistrus* (n = 2), *Coridoras* (n = 4) and *Danio rerio* (Danio, n = 3 pools of 5 fish each). Bars represent means $\pm$ standard deviation. N.D. is not detected. EROD detection limit is 2.5 pmols of resorufin.

Figure 2. Immunodetection of CYP1A protein in control and PCB126 exposed *Pterygoplichthys* by western blotting using the monoclonal antibody MAb 1-12-3

Figure 3. Ethoxyresorufin docking frequencies of *Pterygoplichthys*, *Ancistrus* and *Danio rerio* CYP1A protein models

Black bars represent the ER docking frequency inside the active site. White bars represent the total frequency of ER dockings out of the active site. Frequencies were calculated based on a total of 100 conformations selected by genetic algorithm after 27,000 generations. The grid size was 126x126x126 ÅÅ with 0.375 between any grid points in all simulations.

Figure 4. The two conformations of docked ER inside *Pterygoplichthys* CYP1A active site
a) shows the most frequent (12%) docked conformation, with the ethoxy group far from the heme iron; b) shows the less frequent (5%) and EROD effective conformation with the ethoxy group closer to the heme iron.

Figure 5. Molecular dynamics of the best conformation of ethoxyresorufin docked inside the active site of *Pterygoplichthys*, *Ancistrus* and *Danio rerio* CYP1A protein models

a) Distance, in nm, between the iron atom of the heme group and the methylene carbon; b) Backbone root-mean-squared deviation (RMSD); and c) backbone root-mean-squared fluctuation (RMSF), all after 10 ns simulation for the three models. *Pterygoplichthys* simulation is shown in red, *Ancistrus* in blue and *Danio rerio* in green.

Table 1

The 48 amino acids positions where *Pterygoplichthys*, *Ancistrus* and *Corydoras* CYP1A sequence are not conserved among 27 fish species.

Position	Specie				Other spp.	Remarks
	<i>Pterygoplichthys</i>	<i>Ancistrus</i>	<i>Corydoras</i>			
4	A	A	A		T	c, e
26	T	I	T		L/V	c, e
32	Q	R	R		R	a, e
34	T	T	M		N/F/L/M/S/K	b
44	H	P	P		K/R/Q	c
57	I	I	V		V/F/L/M	b
59	Q	E	E		E/G	a, e
61	A	S	T		G/S/H	d
62	Q	Q	Q		N/S/A/R/G	c
92	G	G	G		S	c, e
94	K	N	S		N/G/S/I/Y/T/L	a, e
98	H	R	R		R/K	a
122	N	N	D		D/E/A	SRS1, b, e
130	S	S	T		T	SRS1, b
134	N	N	G		G	SRS1, b, e
144	H	H	L		L/Y/M	b, e
152	I	I	I		T/S/N	c, e
155	D	D	S		G/A/S	b, e
156	P	P	Q		K/T/S	c, e
158	G	G	G		P/S/A/Q	c
159	Q	E	E		K/E	a, e
163	T	P	A		A/M/V	b, e
166	M	M	E		E	b, e
181	E	E	G		S/D/T/N/K/G	b
188	C	S	G		S/T/R/K/N	d

Position	Species			Other spp.	Remarks
	<i>Pterygoplichthys</i>	<i>Ancistrus</i>	<i>Corydoras</i>		
222	F	F	F	L	SRS2, <i>c, e</i>
247	M	M	L	I/F/Y	<i>c</i>
251	Q	Q	K	T/A/R/K/S	<i>b</i>
252	D	D	T	T/L/S/M/A/E	<i>b</i>
253	I	I	M	M	<i>b</i>
255	I	K	K	K/A/S/N/R	SRS3, <i>a</i>
257	Q	Q	Q	L/K/M/V	SRS3, <i>c, e</i>
261	R	R	R	E/A/D/T/N/G/S/I	SRS3, <i>c, e</i>
265	V	V	T	K/I/Q/T/S/N/E/C/A	SRS3, <i>b</i>
340	V	V	A	H/A/T	<i>b</i>
363	S	T	N	L/R/V/C/T	<i>d</i>
365	L	L	P	S/T	<i>c, e</i>
369	R	L	N	N/K/D/H	<i>b</i>
408	N	N	K	K	<i>b, e</i>
409	N	N	D	D	<i>b, e</i>
448	M	M	I	L/Q/V/A	<i>c</i>
464	V	V	V	I	<i>c</i>
489	P	P	L	M/I/K/L	<i>b</i>
490	G	D	P	P	<i>b</i>
494	P	P	L	L/V	SRS6, <i>b</i>
510	F	F	E	L/H/Q/R/Y/M/N	<i>c</i>
512	C	C	P	R/S/K/G	<i>c</i>
521	V	A	-	-E/K/L/H/C/Q	<i>b</i>

SRS(#): inside or in the border of an SRS, as indicated by the number;

^a substitutions in residues absolutely conserved among a set of 27 species;

^b residues substituted in Loricariidae species but conserved among a set of 27 species;

^c residues substituted in Siluriform species but conserved among a set of 27 species;

^d residues substituted in *Pterygoplichthys* and in *Corydoras* species but conserved among *Ancistrus* and a set of 27 species;

^e nonconservative amino acid change;