CYP27A1 expression in gilthead sea bream (*Sparus auratus*, L.): effects of calcitriol and parathyroid hormone-related protein

Gideon S Bevelander, Elsa S L C Pinto¹, Adelino V M Canario¹, Tom Spanings and Gert Flik

Department of Animal Physiology, Faculty of Science, Radboud University Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands

¹Centre of Marine Sciences (CCMAR), University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

(Correspondence should be addressed to G Flik; Email: g.flik@science.ru.nl)

Abstract

Little is known about vitamin D metabolism in fishes. Several reports have shown hydroxylase activities in various organs to produce vitamin D metabolites, but the enzymes involved have not been isolated or characterized. We isolated and characterized a renal mitochondrial hydroxylase, CYP27A1, that governs vitamin D metabolism in gilthead sea bream, *Sparus auratus*. The enzyme is highly expressed in kidney and to a far lesser extent in liver. When treated with 25-hydroxy vitamin D or calcitriol, the kidney responded differentially

and time dependently with CYP27A1 mRNA expression levels. This response substantiates a role for CYP27A1 in fish vitamin D metabolism. This notion is strengthened by upregulation of CYP27A1 in sea bream treated with parathyroid hormone-related protein (PTHrP), and suggests an original role for PTHrP in calcitriol-regulated processes in fish similar to the role of PTH in mammalian vitamin D-dependent processes.

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Introduction

Vitamin D₃ requires transformations to become bioactive. These transformations involve cytochrome P450 (CYP) enzymes that hydroxylate the steroid. In mammals, the most abundant source of CYP enzymes is the liver, where a first hydroxylation takes place: the 25-carbon is hydroxylated to produce the intermediate 25-hydroxycholecalciferol (Jones *et al.* 1998). The proper enzyme activity responsible for this 25-hydroxylation is still rather enigmatic, even in mammals; several candidate enzymes with 25-hydroxylase ability and broad substrate specificity have been proposed (Aiba *et al.* 2006). One example is the sterol 27-hydroxylase (CYP27A1), which carries out the 25-hydroxylation of vitamin D₃ as well as the 27-hydroxylation of cholesterol in bile acid biosynthesis (Ibarra *et al.* 2004). In mammalian kidney, 25-hydroxycholecalciferol is once more hydroxylated into more potent metabolites, viz. 1 α ,25-hydroxyvitamin D₃ (calcitriol) and 24R,25-dihydroxycholecalciferol; CYP27B1 makes calcitriol and CYP24 produces 24R,25-dihydroxycholecalciferol. It is noteworthy that, CYP24 also uses calcitriol to produce 1 α ,24R,25-trihydroxycholecalciferol, the first product in the C-24 oxidation pathway responsible for degradation of calcitriol.

Comparatively little is known in teleosts concerning 'vitamin D₃ physiology'. Indeed, classical genome-mediated responses to calcitriol have been demonstrated: calcitriol treatments *in vivo* elevate plasma calcium and/or phosphate levels (Larsson *et al.* 2003). Also, differential responses in non-genomic effects of vitamin D₃ metabolites have been

reported, in euryhaline trout kept in either fresh water or seawater (Larsson *et al.* 2003) or between stenohaline species (freshwater carp and seawater cod; Larsson *et al.* 2001). Calcitriol increases active calcium transport in gut of freshwater (FW) fish, but not so in saltwater (SW) fish. The metabolite 24R,25-dihydroxycholecalciferol was reported to decrease intestinal calcium transport in freshwater fish (Larsson *et al.* 2001, 2003).

In mammalian calcium homeostasis, parathyroid hormone (PTH) and calcitriol act synergistically through a strict regulation of CYP27B1 and CYP24 activities. Calcitriol controls its own degradation by induction of CYP24 expression and suppression of CYP27B1 expression; PTH induces CYP27B1 expression and downregulates CYP24 (Jones *et al.* 1998).

The array of endocrines involved in the calcemic control in fish includes PTH-related protein (PTHrP), a key in calcium balance in fish (Abbink & Flik 2007, Guerreiro *et al.* 2007); more recently, *PTH* genes have been demonstrated in zebrafish (Danks *et al.* 2003) and pufferfish (Gensure *et al.* 2004), but our understanding of the role of the peptides coded by these genes in the calcium physiology of fishes awaits further study (Abbink & Flik 2007). A relationship between PTHrP and calcitriol in calcemic control was recently established for sea bream (Abbink *et al.* 2007); it was shown that a decrease of plasma calcitriol levels, due to dietary vitamin D₃ deficiency, result in an adjustment of (hypercalcemic) PTHrP activities in gills and pituitary cells. Apparently, PTHrP and calcitriol fulfill concerted

hypercalcemic functions in fish, reminiscent of those of PTH and calcitriol in higher vertebrates.

Remarkably, little is known about the way vitamin D₃ metabolism takes place in fish. A 25-OHD-1-hydroxylase activity has been detected in several fish species (Henry & Norman 1975) and the production of vitamin D₃ metabolites has been demonstrated *in vivo* and *in vitro* in kidney and liver of trout, carp, halibut species, Atlantic salmon, Atlantic mackerel, and Atlantic cod (Hayes *et al.* 1986, Takeuchi *et al.* 1991, Graff *et al.* 1999). However, even though a 25-OHD-1-hydroxylase activity has been detected, the enzyme itself has never been isolated.

We report here the isolation and characterization of a gilthead sea bream CYP27A1 cDNA, the tissue distribution of gene expression and the effects of vitamin D₃ metabolites and PTHrP on its expression.

Materials and Methods

Fish

Juvenile, male gilthead sea bream (*Sparus auratus* L.) of ~160–180 g were obtained from a commercial source (TiMar, Culturas em Água, Lda., Portugal). The fish were kept in 1000 l stock tanks with running seawater (flow, 250 l/h); the salinity was 36‰ and the water temperature was 22 °C. The fish were kept under natural photoperiod (14 h light:10 h darkness); the fish stock was fed daily commercial pellets (PROVIMI, Alverca, Portugal) at a ratio of 1% of the total estimated body mass per tank. Experiments were carried out in Portugal and approved by the local ethical committee.

Cloning and characterization of CYP27A cDNA from *S. auratus*

A 300 bp cDNA fragment was obtained when attempting to isolate a cDNA for 25-hydroxyvitamin D₃ 1 α -hydroxylase (CYP27B1) from sea bream kidney using degenerate primers (1 α .fw3: 5'-CT (AGC) CT (AG) G (GC) (GCT) GG (AC) GT (GCT) GAC-3'; 1 α .rev3: 5'-(ACT) GA (ACT) GT (GT) G (AC) (AG) TAGTG (AG) CA-3') giving the highest BLASTX score to vitamin D₃-25-hydroxylase (CYP27A) when searching against the GenBank database (Altschul *et al.* 1990). The complete coding sequence of CYP27A was obtained by screening at high stringency (in Church–Gilbert buffer at 55 °C) with the radiolabeled cDNA probe (Rediprime II random labeling kit, Amersham Pharmacia Biosciences) a sea bream kidney cDNA library constructed in Lambda Zap using the UNIZAP vector (Stratagene, La Jolla, CA, USA). After screening 6 × 10⁵ recombinants, six independent clones were autoexcised in pBluescript phagemid using helper phage and sequenced. Two clones were smaller and not further analyzed; the other four were identical to the sequence described here. The putative CYP27A cDNA was identified by searching the GenBank database with BLASTN and BLASTX. The sequence is deposited in EMBL with accession number AM885865.

Tissue distribution of CYP27A

Fish were deeply anesthetized with 2-phenoxyethanol (0.1% v/v; Sigma–Aldrich) and the whole pituitary gland, gill, liver, kidney, head kidney, muscle, foregut, midgut, and rectum were sampled. For mRNA extraction, tissues were flash-frozen on dry ice and stored at –80 °C until further use.

Phylogenetic analysis CYP27A1

Available vertebrate CYP27A1 sequences were taken from the NCBI site (www.ncbi.nih.gov) via the GenBank database. Multiple sequence alignments were accomplished with the ClustalW program from the European Bioinformatics Institute (www.ebi.ac.uk/clustalw/). With the neighbor-joining method, and Mega version 3.1 (Kumar *et al.* 2004), a phylogenetic tree was constructed on the basis of amino acid difference (*P* distance) with 1000 bootstrap replications.

Physiology

Fish (*n* = 8) randomly selected from their stock tank, one group per time point, were placed in cylindrical 500 l tanks (diameter of tank, 0.85 m). Fish were left to acclimatize for at least 1 week; feeding was discontinued 24 h before the start of the experiments.

Vitamin D metabolites 1 α ,25-dihydroxyvitamin D₃ (calcitriol) or 25-hydroxycholecalciferol (25-OHD) were dissolved in a minimal amount of absolute ethanol and diluted in coconut oil. Fish received a single i.p. injection of coconut oil containing calcitriol (25 µg/kg bodyweight (BW)) or 25-hydroxycholecalciferol (50 µg/kg BW) or vehicle only. Fish were sampled 3, 6, and 9 days after injection. In pilots, no effects of these treatments were seen in the first 48 h following injection; therefore 3, 6, and 9 days were considered, in line with similar slow responses to, for example, estrogens in this fish (Bevelander *et al.* 2006).

PTHrP Fish were injected intraperitoneally with saline (0.9% NaCl) or either Fugu PTHrP (1–34; Danks *et al.* 2003) or the recombinant full length sea bream PTHrP (*sb*PTHrP 1–125). For both the forms, the dose was 10 ng/g BW. Controls and treated groups were sampled 6 and 24 h after injection. The choice for these sampling times was based on pilots where maximum effects were seen within 48 h.

Sampling procedure

Fish were deeply anesthetized with 2-phenoxyethanol (0.1% v/v; Sigma–Aldrich), and blood was taken by puncturing the caudal vessels with a 1 ml tuberculin syringe, rinsed with ammonium heparin (Sigma (H-6279) dissolved in 0.9% NaCl (v/v), at 300 units/ml). The fish were then killed by spinal transection and blood was collected. The collected blood was centrifuged at 13 600 *g* for 10 min and the plasma stored

at -20°C until analysis (see below). Next, liver and kidney samples were quickly collected and flash-frozen in liquid nitrogen and stored at -80°C for later analysis of mRNA expression (see below).

Plasma analyses

The 25-OHD concentration in the plasma was measured by a 25-OHD RIA method (IDS Ltd, Boldon, UK). Briefly, two extraction reagents were added to 50 μl standards and samples resulting in the precipitation of serum proteins and extraction of 25-OHD. After centrifugation (10 min at 2000 g), 50 μl extract supernatant were taken from standards and samples. For each sample, supernatant was incubated with ^{125}I -labeled 25-OHD and a specific sheep antiserum (90 min at $18\text{--}25^{\circ}\text{C}$). The bound fraction was separated from the free fraction by a short incubation with Sac-Cel (20 min at $18\text{--}25^{\circ}\text{C}$) and followed by centrifugation (10 min at 2000 g). The supernatant was discarded and the precipitate reflecting the bound fraction was counted in a γ -counter. The bound radioactivity is inversely proportional to the concentration of 25-OHD. The intra- and inter-assay coefficients of variation were 5 and 8% respectively. Stripped plasma spiked with defined levels of 25-OHD gave highly predictable results (data not shown).

CYP27A mRNA expression

Relative expression of CYP27A mRNA was assessed using quantitative RT-PCR. Tissue was homogenized in TRIzol reagent (Invitrogen) and total RNA was extracted according to the manufacturer's instructions. RNA (1 μg) was incubated with 1 U DNase I (amplification grade; Invitrogen) for 15 min at room temperature to remove genomic DNA. To inactivate DNase activity and linearize RNA, 1 μl of 25 mM EDTA was added and the sample incubated for 10 min at 65°C . Next, 1 μg RNA was reverse transcribed (RT) with 300 ng random primers, 0.5 mM dNTPs, 10 units RNase inhibitor (Invitrogen), 10 mM dithiothreitol, and 200 U Superscript II RT (Invitrogen) for 50 min at 37°C .

For quantitative PCR analysis, five times diluted 5 μl RT mix was used as a template in 25 μl amplification mixture, containing 12.5 μl SYBR Green Master Mix (Applied Biosystems Benelux, Nieuwerkerk aan den IJssel, The Netherlands), 3 μl each primer (final concentration

600 nM). The primer sets used in the RT-PCR for elongation factor-1 α (EF-1 α) were: qFWEF-1 α and qRVEF-1 α , for β -actin: qFWACTIN and qRVACTIN, and for CYP27A: qFWCYP27 and qRVCYP27 (Table 1).

All primer sets had an efficiency over 98% and all products showed a single melting transition. After an initial step at 95°C for 10 min, a real-time quantitative PCR of 40 cycles was performed (GeneAmp 7500, Applied Biosystems) with each cycle consisted of 15 s denaturation at 95°C and 1-min annealing and extension at 60°C . The cycle threshold values were determined and the expression was calculated as a percentage of EF-1 α or β -actin (Pfaffl *et al.* 2002). All samples were assayed in duplicate. Both house-keeping genes gave similar results. For clarity, results are presented here only relative to EF-1 α . Non-reverse transcriptase controls were consistently negative.

Chemicals

Calcitriol and 25-OHD were purchased from Sigma (Sigma-Aldrich). Pufferfish (*Takifugu rubripes*) PTHrP (1–34) was synthesized by Genemed Synthesis Inc. (San Francisco, CA, USA). Homologous recombinant parathyroid hormone-related protein sbPTHrP (1–125) was produced in bacteria (Anjos *et al.* 2005).

Statistical analysis

Data are presented as mean $\pm$ s.d. ANOVA was used to assess differences among groups, followed by the Bonferroni test to determine the level of significance (SPSS Windows version 14.0, Chicago, IL, USA). Significance was accepted when $P < 0.05$.

Results

Cloning and characterization of CYP27A cDNA from *S. auratus*

The full length 2793 bp cDNA CYP27A1 sequence containing an open reading frame encoding a protein of 471 amino acids is shown in Fig. 1. The amino acid identity of the sea bream sequence compared with *Xenopus laevis*, human, rat, and mouse is 45, 40, 41, and 39% respectively.

Table 1 Primer sequences for real-time quantitative PCR

Gene	Primer	Sequence	Accession number
EF-1 α	qFWEF-1 α	gtg gag atg cac cac gag tct	AF184170
	qRVEF-1 α	cgt atc cac gac gga ttt cc	
β -actin	qFWACTIN	aag atg acc cag atc atg ttc ga	AF384096
	qRVACTIN	cga tac cag tgg tac gac cag a	
CYP27A	qFWCYP27	tg gct cta caa gtt tgg ctt tga	AM885865
	qRVCYP27	tga acc gca gcg tgt ctt t	

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1  ggcacgagatccaccgtcacaccgctcgacacatggggtccccgggttgctcagctcggg
61  cccccgggtcgctgcgctctgtctccggacactccccggagctccgacacggcggaagga
4                                     M T C R
121 ggctgaacgtccaggcgcgacagctgcggggaagctgaagagcatcgatgacactgcccgg
24  D R V C P P C T G C S S K D T R T R A
181 gacgagctgtgtccaccaccctgtactggctgttcgtcaaaggatagcggacaagagcc
44  M L Q G L Q R N L Y G P I W R S R F G P
241 atgctgcagggtttacagaggaatctttatgggcccacatctggcgctccaggttcggcccg
64  Y D I V N V A T P E L I A Q V I H Q E G
301 tacgacatcgtaatatgtggcgactccagagctgatcgctcaggtgatccatcaggagggg
84  R Y P V R A E L P H W K E Y L D L R G R
361 cgctaccgggtccgagctgagctgcctcactggaagagtacctggacctgagaggacgg
104 A Y G L H V H T G P A W Y N I R S A L N
421 gcttacggtctgcatgtgcacacaggaccggcggtgtacaacatccgcagcgccctgaac
124 P K M L K L R E V T S Y A P I I H Q V V
481 cccaagatgctgaagttgcggaagtgtacttctacgccccgatcatccaccaggtggtc
144 G D L L R R I E L L R S R S Q D Q N T V
541 ggagatctgctgcgacgcatcgactcctccgaagccgagtcaggaccagaacaccgtt
164 S D M A A E L Y K F G F E G I S S I L F
601 tcagatatggctgctgagctctacaagtttggtttgaaggtatatcctccatcttgttt
184 E T R L G C L Q E E I P K D T L R F I T
661 gagaccaggttgggtgctgcaagaggagattcccaaagacagctcggttcatcact
204 A V N N M L T L S E T V V L F P R W S R
721 gccgtcaacaacatgctgacgctgtccgagactgtggtcctcttccctcgctggagccgt
224 G V L P L W N R F V Q A W D D L Y D V A
781 ggcgtcctcccccttggtgaatcgcttcgttcaggcctgggacgacctctacgatgtggcc
244 R T L I D R R V A E I E A Q V C S G E P
841 aggaccctcatcgacaggagagtggctgaaattgaagtcaggtgtgcagtggcgagccg
264 A E G M Y L T Y L L S S D K L S R A E V
901 gcagagggcatgtacctgacctacctgtgtcctctgacaagctgtccagagccgaggtc
284 Y I S L T E L L L G G V D T T S N T L S
961 tacatcagcctcactgaactgctgctgggaggagtgcacacgacctccaacacctgtcg
304 W A L Y H L A K D R S A Q D R L Y S E V
1021 ttggcgctgtaccatttagcgaaggaccgctcggtcaggatcgactgtacagcgaggtg
324 N S V C P D R R E P T T D N L T M M P Y
1081 aactctgtgtgtccggacagacgggagccgaccacagacaacctgacctgatgccttac
344 L K A V I K E T L R L Y P V V P G N G R
1141 ctgaaagccgtcatcaaggagacgctacgtctttatccggtgggtgacctggaatggacga
364 F I S E N E V V E N Y W F P K K T Q F
1201 ttcatcttcggagaatgaggtgggttggaactactgggtcccaaagaagactcagttc
384 H L C H Y A A C H D E M E F V D P E R F
1261 cacctgtgtcactacgcagcctgtcacgatgagatggagtgtgtcgaccggagcgcttc
404 I P D R W L R D V A P S S G H Y R H H P
1321 atccccgatcggtgggtccgcgacgtggcgcccagcagcgccactaccgacaccaccgg
424 Y S F I P F G V G V R A C V G K R V A E
1381 tacagcttcatccattcggtgtcgcgctgcgggcctgtgtggggaagaggggtggcgag
444 M E M Y F A L S R L M Q H Y E V Q L E D
1441 atggagatgtacttcgctctgtccaggttgatgcagcactatgaggtccagctggaagat
464 E A L V V E P K T R T L L I P A K P I N
1501 gaagcttttgctggtggagccaaaactcgaaccctgctcatccctgcgaagccaatcaac
471 L R F L S R A *
1561 ctgcgcttctgtccagagcctgaggagggcgccgctgaggggtcaacacaacacgtcatc
1621 gcttccactccaccaatcacagcccagtcgtgtgtgttttttaaaccttcacaaaat
1681 attttatttgctaacgattaatccagaccaaagtcaaatacttagttactgtacttgag
1741 tacatgtctcaggtattttgtactttactcgagtatttattccctctgacctttctctc
1801 ctcacgttttcaaacaggctcatcacttttagtttgatgtatttgaggggaatgatggat
1861 tatttttatttgccgctattgtacaggatcaatctatctatccgtgcaaaagctaaactgaa
1921 gctaacgtcatattaatataaattcaaacctcagagggagactttcactgtgatactctac
1981 ttttactcaagttaatttgactcgactgattctcttttagtcttttaattggagtgtttat

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Figure 1 (continued)

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2041 ctttgaacttaatgaagttaatgctcagactttgccttctgcacgatcacatgataacat
2101 gataacatatataatcactgcatgtattcagtaacatcctcggttcagcaaagctttcccg
2161 ctggtgaactttgtatttacgcagatttgattcatcggtccttgtttgcaaagaagccgat
2221 gagtcaaagttcgatgagtcacaaatctggtttgacattaataagaaggtgtgttttcagta
2281 gacactcactgttccccctctggttaataaactgatgagtttgaggatttatattaatgttt
2341 aatttgaactttgttcccccttaattgttttttttaattgttaacagaaactttaaggaag
2401 tgggaggaaaggaagtcatttaacagcttttatctgtttcattttaaatcactgttatat
2461 tttataacagccacagctataaactttttcttgacacaagatttattttttacaccaaagc
2521 ttgatttaacgacttaaacacacttttaagagtctaagggaacgacagtggtgtgaagatc
2581 agttttagttatttttagttgaactgttatcgctgtaacactcctgcacagttatctctga
2641 cgatgttctttacagaataaatgaccttttataatgtcgtcttttctaacaatgtgaatattgt
2701 tctcctaaaatgctgtgtctaaaatgtcattaaatttgaaagctgtacacaaaataatat
2761 tcataatatttaattaaaaaaaaaaaaaaaaaaaaa

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Figure 1 Nucleotide and deduced amino acid sequence of *Sparus* CYP27A1. The deduced amino acid sequence is presented above the nucleotide sequence. The start codon is boxed black and the stop codon is indicated by an asterisk (*). The EBL accession number is AM885865.

To compare the sequence of sea bream with that of other vertebrate species, a multiple alignment was made (Fig. 2). The three conserved residues (Lys³³⁶, Lys³⁴⁰, and Arg⁴⁰³) essential for ferredoxin binding are indicated. Furthermore, two other sites are indicated: a well-conserved cysteine⁴²⁹ residue, where heme is covalently bound to the enzyme and a conserved site which is the putative oxygen-binding site.

A phylogenetic tree was constructed using the neighbor-joining method (Fig. 3). Human, mouse, and xenopus CYP27B1 served as an outgroup. Mammalian CYP27A1 sequences are separated from the sea bream CYP27A1 sequence by chicken and xenopus CYP27A1, conform phylogeny. The predicted sequence of zebrafish clusters with the sequence of sea bream, but we do not include these data as the sequence is only predicted. No other fish sequences are known to us at this moment.

Tissue distribution

CYP27A1 gene expression was detected in pituitary gland, gills, intestine, head kidney, and muscle (Fig. 4) with an apparent absence in liver and highest abundance in kidney. The preparations of cDNA were of constant quality judged by consistent and highly reproducible amplification of the house-keeping gene EF-1 α in all samples.

Expression of CYP27A1

In the injection experiments (vitamin D metabolites and PTHrP), only two tissues were studied: liver and kidney. Neither treatment induced CYP27A1 expression to detectable levels in liver (data not shown); however, renal mRNA levels of CYP27A1 were markedly affected by the hormone treatments. The vitamin D metabolite 25-OHD (Table 2) induced a significant downregulation of CYP27A1 expression 3 days following injection; calcitriol did not induce apparent effects at this time point (Fig. 5A). However, 6 days post-injection, both calcitriol and 25-OHD significantly increased CYP27A1 expression (Fig. 5B). No differences in expression were observed 9 days of post-injection.

CYP27A1 mRNA levels were significantly upregulated following injection of PTHrP (1–125) as readily as after 6 h (Fig. 6A). PTHrP (1–34) had a mild but not statistically significant effect. At 24-h post-injection, neither PTHrP (1–34) nor PTHrP (1–125) resulted in changes in CYP27A1 expression (Fig. 6B).

Discussion

This study describes the complete cDNA sequence of CYP27A1 from gilthead sea bream. Quantitative analysis of mRNA expression shows abundant expression of CYP27A1 in the kidney, while liver expression levels are very low, an essentially reversed situation compared with the mRNA expression profiles in mammals. Injection of two different vitamin D metabolites, viz. 25-OHD and calcitriol, had differential and time-dependent effects on the expression of renal CYP27A1, 25-OHD being inhibitory on the shorter term (at 3 days), calcitriol and 25-OHD being stimulatory on the longer term (at 6 days). PTHrP can upregulate CYP27A1 mRNA levels, which suggests a similar role for PTHrP in fish as that of PTH (and PTHrP) in mammalian vitamin D metabolism. Indeed, consensus exists that PTHrP and PTH share common receptors in fish and mammals alike and thus such a finding for PTHrP in fish is not surprising as the PTHrP receptor is expressed in sea bream kidney and many other tissues (Hang *et al.* 2005, Rotllant *et al.* 2006).

Molecular considerations

Mitochondrial cytochrome P450s are monooxygenases receiving their electrons from NADPH via a two-protein redox chain consisting of ferredoxin reductase and ferredoxin. It has been shown that all mitochondrial P450s possess a conserved binding domain involved in the interaction of P450s with their redox partner ferredoxin. There are two lysine residues considered crucial for this binding, and in human P450c27 (= CYP27A1) a third residue, the positively charged arginine at position 403, was found to participate in

Human	1	-----MAALGCARLRWALRG---AGRGLCPHG-----
Rabbit	1	-----MAALGCARLRWALLGPRVAGCGLCPQG-----
Cow	1	-----MNRMCALGSARLRWALLGRRALPGLGSFG-----
Rat	1	-----MAVLSRMRLRWALLDTRVMGHGLCPQG-----
Mouse	1	-----MAAWSRTRLRWILLDFRVVGRGLCPQG-----
Chicken	1	MAEDLDLPWQTFLPIDIQLPRLWPESAPRDAQEAKLAASCASPVLRVTFEPVQFPQKII
Xenopus	1	-----MSRGGLLLKTCRVAVSQGRAVTCGPPASRLHCVP-----
Sparus	1	-----
Human	25	-----ARAKAAIP-----AALPSDKATGAPG-----
Rabbit	28	-----ARAKAAIP-----TALPADEAAQAPG-----
Cow	31	-----ARAKAAIP-----SALPAAQAAEAPG-----
Rat	28	-----ARAKAAIP-----AALRDHISTEGPG-----
Mouse	28	-----ARAKATIP-----AALQAQESTEGPG-----
Chicken	61	DQRAWHESRSPLRHPGRSVSPVPVPPCLSSSDTCAGCAGCERACIFSDPSEDTRLQPPIG
Xenopus	35	-----QGSGYLQAGRGVS-----VSQGRAVTGAA-----
Sparus	1	-----MTCRDTRVCPEP-----
Human	46	-----AGPGVRRRQSRLEEIPRLGQLRFFFQLFVQGYALQLHQLQVLYKAKYG
Rabbit	49	-----AGPGDRRRRSLEEIPRLGQLRFFYQAFVQGYLLHLHLQVNLKAKYG
Cow	52	-----TGPGDRR--LRSLDELSGPGQLRLFLQLVQGYVLHLHLQVNLKAKYG
Rat	49	-----TCQ--DRPRLRSALDELPGPCTLRFLFQLFTRGYVLHLHLQVNLKAKYG
Mouse	49	-----TCQ--DRPRLRSPAELPGTCTLQFLFQLFLOGYVLHLHLQVNLKAKYG
Chicken	121	PEPPCRTGFSAAAAAGPARLKGPAELPGPGLLRTFVWLFTRGYLLHLHLQVNLKAKYG
Xenopus	59	-----VETADGRKEMKEFDLPGPSLLKNLYYYFVRGYLLHLHLQVNLKAKYG
Sparus	12	-----CTGCSSKDTTRIRAMLOG-----LQRNLYG
Human	94	PMWMSYLGPMH--VNLASAPLLEQVMRQ--KYPVRNDMLWKEHRDQDLTYGPFTTEG
Rabbit	97	PMWMSYLGPMH--VNLASAPLLETVMRQEGKYPVRNDMLWKEHRDHQDLAYGVFTTDG
Cow	99	PIWNRVGPQMH--VHLASAPLLEQVMRQEGKYPVRNDMLWKEHRDQGLSYGPFTTMG
Rat	96	PMWMTTFGTRTN--VNLASAPLLEQVMRQEGKYPTRDSMELWKEHRDHKGLSYGIFTTQG
Mouse	96	PMWMTSFGTYTN--VNLASAPLLEQVMRQEGKYPTRDHMDQWKDHRDHKGLTYGIFTAQG
Chicken	181	PIWKRTEFVLYQN--INIGSPVYLEQLLRQEGKYPMRSDMALWKEHRDTRLPGPFTTEX-
Xenopus	108	PIWKRSEIGKYKM--VNIGDPEALQQLLRQEGKYPMRNKEDLWKAHRDQRLAYGPFTTEG
Sparus	36	PIWRSRFGPYFTDITVNVATPELIAQVTHQEGRYPVRAELPHWKEYLDLRCRAYGLHVHTG
Human	150	HHWYQLRQALN--QRLLKPAAEALYTDAFNEVIDDFMTRLDQLRAESASGNQVSDMAOLF
Rabbit	155	HDWYQLRQALN--QRLLKPAAEALYTDAFNEVIDSFVRLDQLRAESASGDQVPDMADLL
Cow	157	EQWYRLRQTLN--QRMLKPAAEALYTDAFNEVIDFMDQLRQRAESASGDHPVDIAHQF
Rat	154	QQWYHLRHSLN--QRIVKPAAEALYTDAFNEVIDSFTRLDQVRTESASGDQVPDVANLL
Mouse	154	EQWYHLRQALK--QRLLKPDEAALYTDAFNEVIDSFITRLDQVRAESESGDQVPDMAHLL
Chicken	238	---VRPRSDVG--MRIVEPSEAVLYADATGAVVSDLMVRLRDERRCSPSGVLVGDVANLL
Xenopus	166	YHWYRIRSVLN--KMKLPSEASSYAGGINEVVTDFMNLQYMRKASPSGDMVNDVANAL
Sparus	96	PAWYNIRSAFNTPKMLKLRVTSYAPIIHQVVGDLRLRLRLRSRSQDQNTVSDMAABL
Human	208	YFFALEAICYILF--EKRIGCLQRSIPEDTVTFVRSIGLMEQNSLYATFLPKWTRPVLFP
Rabbit	213	YHFALEAICYILF--EKRIGCLQASIPKDTENFIRSVGLMEQNSVYVTFPLPKWTRPPLPF
Cow	215	YFFALEAICYILF--EKRIGCLERSIPKDTETVFSVGLMFHNSLFVTFLETFWTRPPLPF
Rat	212	YHFALEAICYILF--EKRIGCLQRSIPEDTATFIRSVGLMEQNSVYVTFPLKWSRPLLPF
Mouse	212	YHFALEAICYILF--EKRIGCLQPSIPEDTAAFIIRSVGLMEQNSVYVTFPLKWSRPLLPF
Chicken	293	YRFALEGISYILF--ETRIGCLKQVPPETQHFIDSINLMFKNSIFATVLPFRWSRKVLFP
Xenopus	224	YRFALEGISYILF--ETRIGCLKQTPPETQKFIDSIGYMEQNSVYVTFPLQWTKGILP
Sparus	156	YKFCFEGISSILFTFETRIGCLQEEIPKDTLRFITAVNNMLTLSETVVLFPFRWSRGVLEL

Figure 2 (continued)

Figure 2 Alignment of CYP27A1s for eight vertebrate species. Hyphens indicate gaps that were introduced to achieve maximum identity. Identical amino acids are indicated in black boxes, conservative substitutions in gray. The two arrows close to each other indicate the two lysine residues and the third arrow indicates the arginine residue. A conserved cysteine residue, the ligand for the heme group, is indicated in an underlined block and a putative oxygen-binding site is indicated (doubly underlined). Accession numbers: human (*Homo sapiens*), NP_000775; rabbit (*Oryctolagus cuniculus*), P17177; cow (*Bos taurus*), XP_883724; mouse (*Mus musculus*), NP_077226; rat (*Rattus norvegicus*), NP_849178; chicken (*Gallus gallus*), XP_422056; and Xenopus (*Xenopus laevis*), AAH77308.

binding as well, making the bond tighter (Pikuleva *et al.* 1999). These three conserved residues are found in all vertebrate CYP27A1 sequences (Fig. 2) and indeed must be highly relevant for the functioning of the enzyme. The

conserved cysteine⁴²⁹ responsible for heme binding and a conserved site for oxygen binding are found not only in all known CYP27A1s but also in other vitamin D-related cytochromes (Jones *et al.* 1998). This taken together with

Figure 3 Phylogenetic tree of CYP27A1 sequences constructed using the neighbor-joining method. Numbers at the branch nodes represent the confidence levels of 1000 boot strap replications. Human, mouse, and *Xenopus tropicalis* (*Xenopus* CYP27B1) CYP27B1 served as an outgroup. Accession numbers as in Fig. 2. Additional accession numbers: human (*Homo sapiens*), NP_000776; mouse (*Mus musculus*), NP_034139; and *Xenopus* (*Xenopus tropicalis*), NP_001006907.

significant yet moderate overall amino acid homology (homology for consensus sequences within the protein such as the heme- and the oxygen-binding regions are very much higher indeed) and the clustering in phylogenetic analyses, confirms the identity of the sea bream enzyme presented here as a mitochondrial CYP27A1.

25-Hydroxylation

The mammalian liver and several extrahepatic tissues possess mitochondrial enzyme activities that hydroxylate the

25-carbon of vitamin D₃ and other steroids (Bhattacharyya & DeLuca 1974, Bjorkhem *et al.* 1980). The presumed enzyme was first identified as a broad spectrum sterol 27-hydroxylase with a designated role in hepatic bile acid biosynthesis, catalyzing the first step in the oxidation of the cholesterol side chain in the ‘acidic’ bile biosynthesis pathway (Cali & Russell 1991). Consensus has been reached now that an extrahepatic CYP27A1 serves this role in cholesterol homeostasis and efficient clearance of cholesterol (Babiker *et al.* 1997). Further support for extrahepatic 25-hydroxylase activity came from studies that show expression of CYP27A1 in the brain, vascular endothelium, kidney, and liver (Andersson *et al.* 1989, Reiss *et al.* 1997); a situation that, with the exception of what we describe here for the liver, is comparable with that in sea bream tissues.

Figure 4 Expression of Sparus CYP27A1 as determined by quantitative RT-PCR. Data are presented as mean ± s.d. (N=5).

Table 2 25-OHD plasma levels (nM) following 25-OHD and calcitriol treatment of seawater sea bream

	Vehicle	25-OHD	Calcitriol
Time (days)			
3	31.2 ± 2.20	51.0 ± 9.96*	36.6 ± 4.09
6	33.8 ± 4.72	59.9 ± 11.75*	36.4 ± 3.29
9	33.4 ± 3.01	47.7 ± 9.11*	31.6 ± 3.11

Seawater sea bream were injected with vehicle or hormones (see Materials and Methods) and sampled for blood 3, 6, and 9 days later. Plasma was analyzed for 25-OHD; vehicle-treated fish did not differ in time nor did calcitriol affect 25-OHD levels compared with vehicle-treated fish; 25-OHD-injected fish had elevated plasma levels of 25-OHD (*P<0.05; n=9) for the duration of the experiment.

Figure 5 Kidney CYP27A1 expression after (A) 3, (B) 6, and (C) 9 days, relative to the house-keeping gene EF-1 α , in control and sea bream injected with calcitriol (25 μ g/kg BW) or 25-hydroxycholecalciferol (50 μ g/kg BW), quantified by real-time PCR. Different letters denote significant differences at $P < 0.05$.

1 α -Hydroxylation

Henry & Norman (1975) were the first to demonstrate a vitamin D₃ hydroxylase *in vitro* in renal tissue of a full series of vertebrates including fresh- and seawater fishes. Subsequent studies showed hydroxylation of vitamin D₃ and 25-OHD

Figure 6 Kidney CYP27A1 expression after 6 and 24 h, relative to the house-keeping gene EF-1 α , in control and sea bream injected with Fugu PTHrP (1-34) or PTHrP (1-125; 10 ng/g BW), quantified by real-time PCR. Letters denote significance at $P < 0.01$.

into their polar metabolites in liver and kidney (Hayes *et al.* 1986, Bailly du Bois *et al.* 1988, Takeuchi *et al.* 1991). The demonstration of 1 α -hydroxylase activity in liver in carp and bastard halibut (Takeuchi *et al.* 1991), and Atlantic cod (Sundell *et al.* 1992) led to the postulation that also in these species the liver is a site for the first hydroxylation of vitamin D₃. However, hydroxylase activity was demonstrated in extrahepatic (and extrarenal) sites in a variety of fishes, and this suggests both that hydroxylations at the 1 and 25 positions outside the liver may occur to produce calcitriol (Graff *et al.* 1999). Considering the situation in mammals with a possible involvement of CYP27B1 in calcitriol metabolism, a further search for analogous hydroxylation pathways in fish seems warranted.

In the mammalian kidney, CYP27B1 hydroxylates 25-OHD to produce circulating calcitriol; remarkably, renal CYP27A1 may also catalyze 1 α -hydroxylation (Postlind *et al.* 2000, Araya *et al.* 2003), and this implies that CYP27A1 may convert vitamin D₃ into calcitriol.

In sea bream, just as in higher vertebrates, the expression of CYP27A1 is seen in a variety of tissues; remarkably,

the highest expression is observed in kidney and, from our quantitative mRNA analysis, little or none in liver. Also, and in contrast to reports on mammals, we could not alter liver CYP27A1 expression by 'classical' hormonal treatments, while renal CYP27A1 expression markedly responded to both calcitriol and 25-hydroxycholecalciferol. We conclude that the fish kidney may be the prime and possibly unique site for calcitriol formation, a situation clearly different from the two-step process in liver and kidney observed in mammals.

Our findings contrast with those of Takeuchi *et al.* (1991), who demonstrated that in carp and bastard halibut 25-hydroxylase activity is essentially confined to a hepatic microsomal fraction, and 1 α -hydroxylase activity to renal and hepatic mitochondrial fractions (Takeuchi *et al.* 1991), which favors either a liver–renal or liver–liver pathway for dihydroxylation of vitamin D₃. Very low-expression levels of the mitochondrial CYP27A1 were found in sea bream liver and no effects could be established of vitamin D metabolite treatments and, therefore, we doubt whether in sea bream the liver plays a role in calcitriol formation; this would imply a renal–renal pathway for dihydroxylation in this species. Clearly, in fish, calcitriol production pathways may differ.

Responses to hormone treatments

The 25-OHD treatment predictably elevated plasma level of this metabolite reaching its maximum 6 days post-injection. Under this 25-OHD regimen, CYP27A1 expression showed an initial downregulation at day 3 followed by a transient upregulation at day 6. A plethora of receptors and pathways may be involved in regulation following 25-OHD treatment; to establish the factor responsible for the upregulation seen here requires more research (Langmann *et al.* 2005, Tang 2007, personal communication). We speculate that injection of 25-OHD elevated calcitriol levels (unfortunately, we were not able to assess plasma calcitriol levels) and by doing so enhanced expression of CYP27A1 (for calcitriol effects, see below).

Calcitriol injection was without effect after 3 days; however, after 6 days a twofold upregulation could be seen as in the 25-OHD-treated animals. In mammals, downregulation by calcitriol on CYP27A1 has been reported (Theodoropoulos *et al.* 2003), but the pattern we show here parallels data on Atlantic salmon: it was shown that calcitriol injections result initially in a drop of plasma calcitriol 3 days after the injection and then return to normal levels (on 5 and 7 days of post-injection); these findings suggest a clearance of the exogenous calcitriol and a suppression of the endogenous production of calcitriol in the first days of post-injection by downregulation of its hydroxylase, followed by an upregulation restoring basal levels. Such effects were also observed in salmon treated with calcitriol (Flik *et al.* unpublished).

The involvement of CYP27A1 in sea bream vitamin D metabolism is further supported by the upregulation of the gene when fish are subjected to PTHrP. Interactions of the vitamin D system and PTHrP in fishes are essentially

unknown, with the exception of one study in which the calcium regulation in vitamin D-deficient sea bream was studied and a relationship was found between PTHrP and calcitriol (Abbink *et al.* 2007). In humans, when exogenous PTHrP or PTH is administered, calcitriol levels tend to rise (Fraher *et al.* 1992, Everhart-Caye *et al.* 1996). Moreover, in a study looking at the vitamin D metabolism in a rat model for humoral hypercalcemia of malignancy, it was established that PTHrP enhanced 1 α -hydroxylase expression in bisphosphonate-treated rats (Michigami *et al.* 2001). PTHrP is a well-established hypercalcemic hormone in sea bream and thus we tentatively conclude that PTHrP may well be involved in CYP27A1 regulation, may be to evoke hypercalcemia via CYP27A1-dependent calcitriol production to control calcium uptake. Interestingly, a similar interrelationship between PTHrP and hypercalcemic actions of estrogen was established in this fish (Guerreiro 2002).

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