Androgen metabolism via 17β-hydroxysteroid dehydrogenase type 3 in mammalian and non-mammalian vertebrates: comparison of the human and the zebrafish enzyme

R Mindnich, F Haller, F Halbach, G Moeller, M Hrabé de Angelis and **J Adamski**

GSF-National Research Center for Environment and Health, Institute of Experimental Genetics, Genome Analysis Center, Ingolstaedter Landstr. 1, 85764 Neuherberg, Germany

(Requests for offprints should be addressed to J Adamski; Email: adamski@gsf.de)

Abstract

Formation and inactivation of testosterone is performed by various members of the 17β-hydroxysteroid dehydrogenase (17β-HSD) family. The main player in testosterone formation is considered to be 17β-HSD type 3, which catalyzes the reduction of androstenedione to testosterone with high efficiency and is almost exclusively expressed in testis. So far, only the mammalian homologs have been characterized but nothing is known about the role of 17β-HSD type 3 in other vertebrates. In this study, we describe the identification and characterization of the zebrafish homolog. We found zebrafish 17β-HSD type 3 to be expressed in embryogenesis from sphere to 84 h post-fertilization. Expression was also detected in various tissues of both male and female adults, but displayed sexual dimorphism. Interestingly, expression was not highest in male testis but in male liver. In female adults, strongest expression was observed in ovaries. At the subcellular level, both human and zebrafish 17β-HSD type 3 localize to the endoplasmic reticulum. The zebrafish enzyme *in vitro* effectively catalyzed the conversion of androstenedione to testosterone by use of NADPH as cofactor. Among further tested androgens epiandrosterone and dehydroepiandrosterone were accepted as substrates and reduced at C-17 by the human and the zebrafish enzyme. Androsterone and androstanedione though, were only substrates of human 17β-HSD type 3, not the zebrafish enzyme. Furthermore, we found that both enzymes can reduce 11-ketoandrostenedione as well as 11β-hydroxyandrostenedione at C-17 to the respective testosterone forms. Our results suggest that 17β-HSD type 3 might play slightly different roles in zebrafish compared with human although testosterone itself is likely to have similar functions in both organisms.

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Introduction

Androgens are a group of hormones considered to be involved in many different aspects of sexual and especially male development. Testosterone plays a central role among the androgens because it binds strongly to the androgen receptor, is a substrate for the 5α-reductase giving rise to the most potent androgen dihydrotestosterone and is a substrate for the aromatase yielding estradiol (for a review on steroid hormone formation see also Payne & Hales 2004). The formation of testosterone from androstenedione as well as the respective back-reaction is catalyzed by enzymes of the family of 17β-hydroxysteroid dehydrogenases (17β-HSDs). Structurally, nearly all 17β-HSDs belong to the short-chain dehydrogenase/reductase (SDR) superfamily. In human, mouse and rat, the reduction of androstenedione to testosterone is catalyzed by 17β-HSD type 3 (Geissler *et al.* 1994, Tsai-Morris *et al.* 1999) and type 5 (Dufort *et al.* 1999, Rheault *et al.* 1999, Luu-The *et al.* 2001). In rodents, additionally 17β-HSD type 1 catalyzes this reaction very efficiently (Akinola *et al.* 1996, Nokelainen *et al.* 1996). In mammals, testicular formation of testosterone seems to be mainly governed by 17β-HSD type 3, which has been shown to be highly and almost exclusively expressed in the testis of human (Geissler *et al.* 1994), mouse (Mustonen *et al.* 1997, Sha *et al.* 1997) and rat (Tsai-Morris *et al.* 1999). The enzyme is mainly involved in male sexual development resulting in male pseudohermaphroditism upon loss-of-function mutations (Geissler *et al.* 1994).

In fish, androgens were shown to play important roles in gonadogenesis (Bhandari *et al.* 2004) and spermatogenesis (Miura *et al.* 1991, Loir 1999) as well. The latter aspect seems to be closely connected to 11-ketotestosterone, assuming a special role for this modified androgen in fish sexual maturation. Furthermore, the role of such modified together with the so-called classical androgens on mating and nesting behavior of fish has been studied in some selected species (Brantley *et al.* 1993, Oliveira *et al.* 2002, Pall *et al.* 2002). In contrast to mammals, especially the molecular mechanisms underlying androgen function in fish are still widely unknown. The androgen receptors from

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several different fish species have been cloned and characterized at the molecular level (Ikeuchi *et al.* 1999, Sperry & Thomas 1999, Takeo & Yamashita 1999, Touhata *et al.* 1999, Kim *et al.* 2002, Wilson *et al.* 2004) and central enzymes of the general pathway leading to the formation of steroid hormones, for example P450 cholesterol side-chain cleavage enzyme (Lai *et al.* 1998, Hsu *et al.* 2002) and 17α-hydroxylase/17,20-lyase (Sakai *et al.* 1992, Trant 1995, Kazeto *et al.* 2000*b*), have been identified as well. Concerning enzymes of the 17β-HSD family though, only 17β-HSD type 1 from eel (Kazeto *et al.* 2000*a*) and zebrafish (Mindnich *et al.* 2004) have been studied in detail and in both species the enzyme does not seem to be involved in androgen metabolism. To get more insight into the molecular regulation of androgen function in fish, we identified and analyzed 17β-HSD type 3 in zebrafish with respect to its expression pattern and enzymatic activity.

Materials and methods

Identification of zebrafish 17β-HSD type 3

Identification of the zebrafish 17β-HSD type 3 homolog was carried out by comparison of the human and mouse protein sequences to the zebrafish expressed sequence tag (EST) database at NCBI using the tBLASTn tool (www.ncbi.nlm.nih.gov). EST sequences were selected as putative homologs if either they were already annotated as similar to 17β-HSD 3 or had an alignment score of >80 bits when their complete putative coding sequence was aligned to the mammalian homologs in the SwissProt database using BLASTx. In this way, two candidate clones were identified and retrieved from the Resource Center, Primary Database (RZPD) of the German Human Genome Project at the Max-Planck-Institute (Berlin, Germany): IMAGp998 N0614301Q3 and IMAGp998 G1614299Q3. Upon sequencing (carried out by SequiServe, Vaterstetten, Germany), the first of these EST clones could be shown to contain the full-length coding sequence while the latter displayed some nucleotide exchanges and lacked the sequence corresponding to exon 2. Only the first clone, IMAGp998N0614301Q3, was considered for further analysis and the annotated sequence was submitted to the GenBank Nucleotide Sequence Database under accession no. AY551081.

Phylogenetic analysis

The datasets were created by retrieving related sequences from three different sources: a psi-BLAST (Altschul *et al.* 1997) of the mouse 17β-HSD 3 protein sequence against the non-redundant protein database at NCBI; a BLink-link sequence of the mouse protein entry in the NCBI database (NP_034605 and NP_032317); a translated BLAST sequence (tBLASTn) of the zebrafish 17β-HSD 3 protein sequence against EST databases of *Ciona intestinalis*, *Caenorhabditis elegans* and *Drosophila melanogaster*. Sequences were aligned by ClustalW (Thompson *et al.* 1994; www2.ebi.ac.uk/clustalw) and, to avoid redundancy, the alignment was monitored and manually edited in BioEdit (www.mbio.ncsu.edu/BioEdit/bioedit.html). Phylogenetic analyses were conducted with MEGA version 2·1 (Kumar *et al.* 2001 and references therein; www.megasoftware.net) using the neighbor-joining algorithm and 1000 bootstraps. Accession numbers of all selected sequences are as follows: apSPM2 (O57314), bn3 KAR (AAO43448·1), btpSDR (AV601497 and CK836287), ceNP505205 (NP_505205·1), ceNP506448 (NP_506448·1), ceNP506449 (NP_506449·1), ciAK116587 (AK116587·1), ciAL669220 (AL669220·1), ciBW274894 (BW274894·1), dmCG6012 (NP_609817·1), dmRE48687p (AAN71421·1), ggSDlike (BU137375), hsHSD17B12 (NP_057226·1), hsHSD17B3 (NP_000188·1), hsSDlike (NP_113651·3), hv3 KAR (AAB827661·1), mmHSD17B12 (NP_062631·1), mmHSD17B3 (NP_032317·1), mmSDlike (CA315224 and BE865064), rnHSD17B3 (NP_446459·1), ssSDlike (BP142073 and BX671798), xlHSD17B12 (AAH41194·1), xlpSDR (BC074162·1), zfHSD17B12A (AAS58452), zfHSD17B12B (AAS58450), zfHSD17B3 (AAS58451), zfpSDR (AAP74564) and zm3 KAR (AAB82767·1).

RNA preparation and reverse transcriptase (RT)-PCR

Preparation of RNA from zebrafish embryos and adult fish (AB wild-type strain) as well as the reverse transcription into cDNA was performed as described by Mindnich *et al.* (2004). For RT-PCR, 1 μl cDNA was added to a total reaction volume of 20 μl (1·5 mM $MgCl_2$ and 2·5 U Taq polymerase (New England Biolabs)) and the PCR run on a Robo-Cycler (Stratagene) with one cycle of 3 min at 95 °C and 35 cycles of 30 s at 95 °C, 30 s at 55 °C and 1 min at 72 °C. Zebrafish actin (forward, 5′-CTGGTTGTTGACAACG GATCCG-3′; reverse, 5′-CAGACTCATCGTACTCC TGCTTGC-3′) was amplified to monitor cDNA quality. Expression of zebrafish 17β-HSD 3 was investigated by use of the following primer pair: forward, 5′-AAACAT CGAGGGATTGGATATTGGC-3′; reverse, 5′-TGGC TTCTGATGTCCTGTCATTGC-3′. Each PCR was conducted three to five times including controls without template (water-control) to assure reproducibility.

Cell cultures

All media were supplemented with 10% fetal calf serum (Biochrom), 100 units/ml penicillin G-sodium salt (Invitrogen) and 100 μg/ml streptomycin sulphate

(Invitrogen). HeLa cells (ACC57; Deutsche Sammlung von Mickroorganismen und Zellkulturen, Braunschweig, Germany) were maintained in RPMI 1640 medium (Invitrogen) at 37 °C and 5% CO_2. HEK-293-Ebna cells (Invitrogen) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum at 37 °C and 5% CO_2. Zebrafish zf4 cells (Driever & Rangini 1993; LGC Promochem, Germany) were grown in a 1:1 (v/v) mixture of Dulbecco's (Wessel, Germany) modified Eagle's medium/Ham's F12 medium (Invitrogen) at 28 °C and 5% CO_2.

Activity measurements

For activity measurements, the human and zebrafish full-length coding sequences of 17β-HSD 3 were cloned into the pcDNA3 vector (Invitrogen). Transient transfection of cells in 250 ml cell-culture flasks with 8 μg DNA complexed with 24 μl FuGENE transfection reagent (Roche) in 800 μl medium were carried out as recommended by the supplier. 24 h after transfection, cells were harvested, counted, split into aliquots and the cell pellets frozen at −80 °C until the same lot was subjected to activity measurements with different substrates and cofactors. The aliquots contained 8×10^5 cells in the case of zf4 cells and 3×10^6 cells in case of HEK-293-Ebna cells.

HPLC

One aliquot of frozen cells per measurement was thawed and resuspended in 100 μl reaction buffer (100 mM sodium phosphate, 0·05% BSA and 1 mM EDTA, pH 7·4). 400 μl reaction buffer containing 18·7 nM testosterone (1,2-^{3}H(N); Perkin Elmer) or 13·5 nM androstenedione (1,2,6,7-^{3}H(N); Perkin Elmer) were added. Reactions were started by addition of 50 μl cofactor (5 mg/ml in reaction buffer). After 90 min incubation at 37 °C the reaction was stopped by addition of 100 μl stop solution (0·21 M ascorbic acid in 1% acetic acid in methanol). Samples were extracted on Strata C_{18}-E reverse-phase columns (Phenomenex) and eluted twice with 200 μl methanol. Separation of educt and product in a 20 μl sample was performed through HPLC (Beckman) running isocratic water/acetonitrile (55:45, v/v) on a reverse-phase LUNA 5μ C_{18} (2) column (Phenomenex) at a flow rate of 1 ml/min. Detection of the tritiated steroids proceeded with an HPLC radioactivity monitor after mixing with scintillation solution (Ready Flow III; Beckman). Integration of the peaks in the HPLC spectra by use of the 32 Karat software (Berthold) yielded the percentage of steroid conversion.

TLC

Per measurement, two aliquots of frozen cells were thawed and resuspended in 100 μl reaction buffer (100 mM sodium phosphate, pH 7·4, 0·05% BSA and 1 mM EDTA). 900 μl reaction buffer and 5 μl of the respective steroid (10 mM) were added and the reaction was started by addition of 50 μl cofactor (5 mg/ml in reaction buffer). Following 4 h of incubation at 37 °C, samples were processed by reverse-phase extraction on Strata C_{18}-E columns as described above. Eluates were vaccum-dried and the steroids resuspended in 30 μl chloroform. Suspensions were spotted on a TLC plate (silica gel 60 F_{254}; Merck) and vials rinsed with 20 μl chloroform, which was also applied to the TLC plate. Steroids were separated in chloroform/methanol (95:5, v/v) and detected by spraying with ethanol/H_2SO_4 (70:30, v/v) followed by incubating at 140 °C for 4–6 min.

Subcellular localization studies

For localization studies, the human and zebrafish full-length coding sequences of 17β-HSD 3 were cloned into different LivingColor™ vectors (Clontech) resulting in fluorescent protein-tagged constructs. N-terminal tags were achieved by subcloning into pEGFP-C2; C-terminal tags were constructed by cloning into pEYFP-N1 in the case of the human protein and pECFP-N1 in the case of the zebrafish protein. About 10^7 cells were plated out in six-well plates containing glass coverslips and transiently transfected with 2 μg DNA/6 μl FuGENE6 as recommended by the supplier (Roche). Medium was changed 6 h after transfection and cells were studied 24 h later. For staining of the endoplasmic reticulum (ER), cells were incubated under growth conditions with fresh medium supplemented with 500 nM ER-Tracker Blue/White (Molecular Probes) for 30 min followed by 5 min incubation at room temperature with fresh medium without dye. Cells were then fixed for fluorescence microscopy by incubation in medium containing 3·7% formaldehyde for 30 min at growth conditions. Cells were washed twice in PBS and once in distilled water and mounted on slides with VectaShield mounting medium (VectorLabs). Samples were analyzed by confocal laser-scanning fluorescence microscopy on a LSM510 Meta microscope with a 63× oil-immersion objective.

Results

Identification of zebrafish 17β-HSD 3

In search of zebrafish 17β-HSD 3 we performed *in silico* screens on the zebrafish EST database at NCBI with the sequence of the human protein as a probe. Four candidates were retrieved that revealed 40–50% amino acid identity to the human protein. Three of these zebrafish proteins had already been identified in a previous search for 17β-HSD 3 and were demonstrated

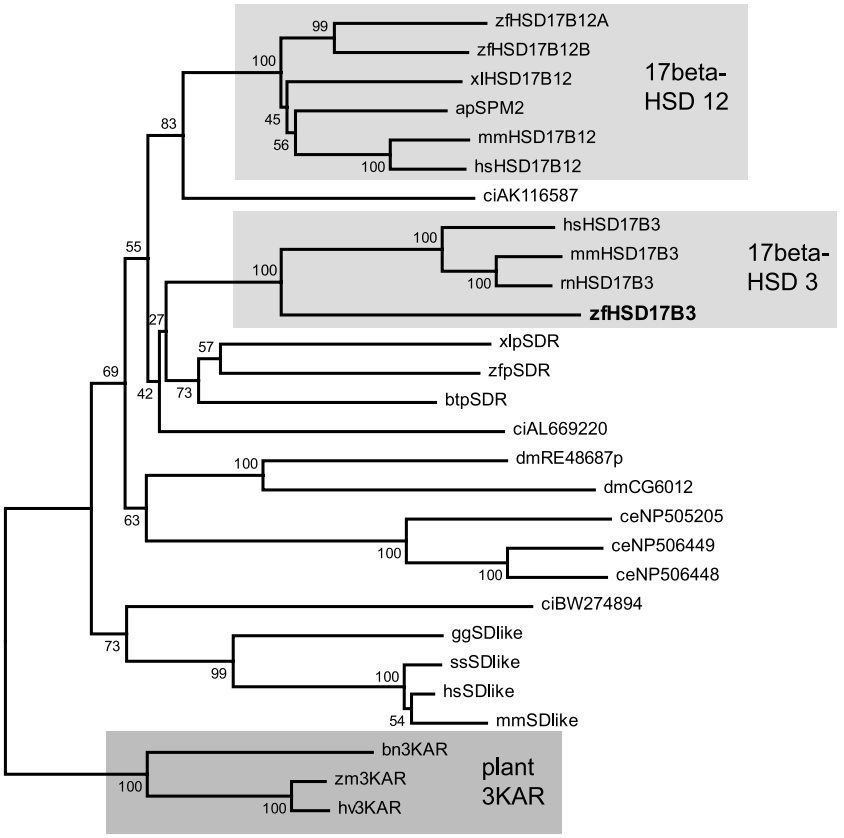


Figure 1 Evolutionary context of zebrafish 17β-HSD 3.Vertebrate and invertebrate sequences with a relation to the group of 17β-HSD 3 were analyzed. The dendrogram was calculated with a neighbor-joining algorithm applying 1000 bootstraps and was rooted by taking plant 3-keto acylreductases (3 KARs) as the outgroup. The numbers given at branches correspond to percentage of bootstraps supporting this particular dichotomy. The zebrafish enzyme analyzed in this report is highlighted in bold. A complete list of the sequences employed for tree calculation can be found in the Materials and methods section. The first two letters denote the organism, as follows: ap, *Anas platyrhinchos*; bn, *Brassica napus*; bt, *Bos taurus*; ce, *Caenorhabditis elegans*; ci, *Ciona intestinalis*; dm, *Drosophila melanogaster*; gg, *Gallus gallus*; hs, *Homo sapiens*; hv, *Hordeum vulgare*; mm, *Mus musculus*; rn, *Rattus norvegicus*; ss, *Sus scrofa*; xl, *Xenopus laevis*; zf, zebrafish; zm, *Zea mays*.

not to be the zebrafish homolog (Mindnich *et al*. 2004). The fourth protein derived from the screen was a new candidate and was thus investigated further. For this sequence, two clones could be ordered directly from RZPD and sequenced, identifying the full-length coding sequence of the putative zebrafish 17β-HSD 3.

Phylogenetic analysis

Identification of the complete coding sequence of putative zebrafish 17β-HSD 3 confirmed the close relation to the mammalian homologs by amino acid identities of 45, 44 and 46% to the human, mouse and rat enzymes, respectively. To investigate whether the candidate was indeed the zebrafish 17β-HSD 3 homolog, a phylogenetic analysis was performed. The resulting dendrogramm (Fig. 1) revealed a variety of subgroups of different evolutionary origins and a clear separation of 17β-HSD 3 and 17β-HSD 12, supported by high bootstrap values. The zebrafish candidate clearly falls into the group of 17β-HSD 3. Due to this result and further findings (see below), the full-length coding sequence was submitted to the GenBank Nucleotide Sequence Database at NCBI as zebrafish 17β-HSD 3 (under accession no. AY551081).

Motifs and amino acid identities

An alignment of the zebrafish protein with the human, mouse and rat homologs (Fig. 2) reveals the functionally

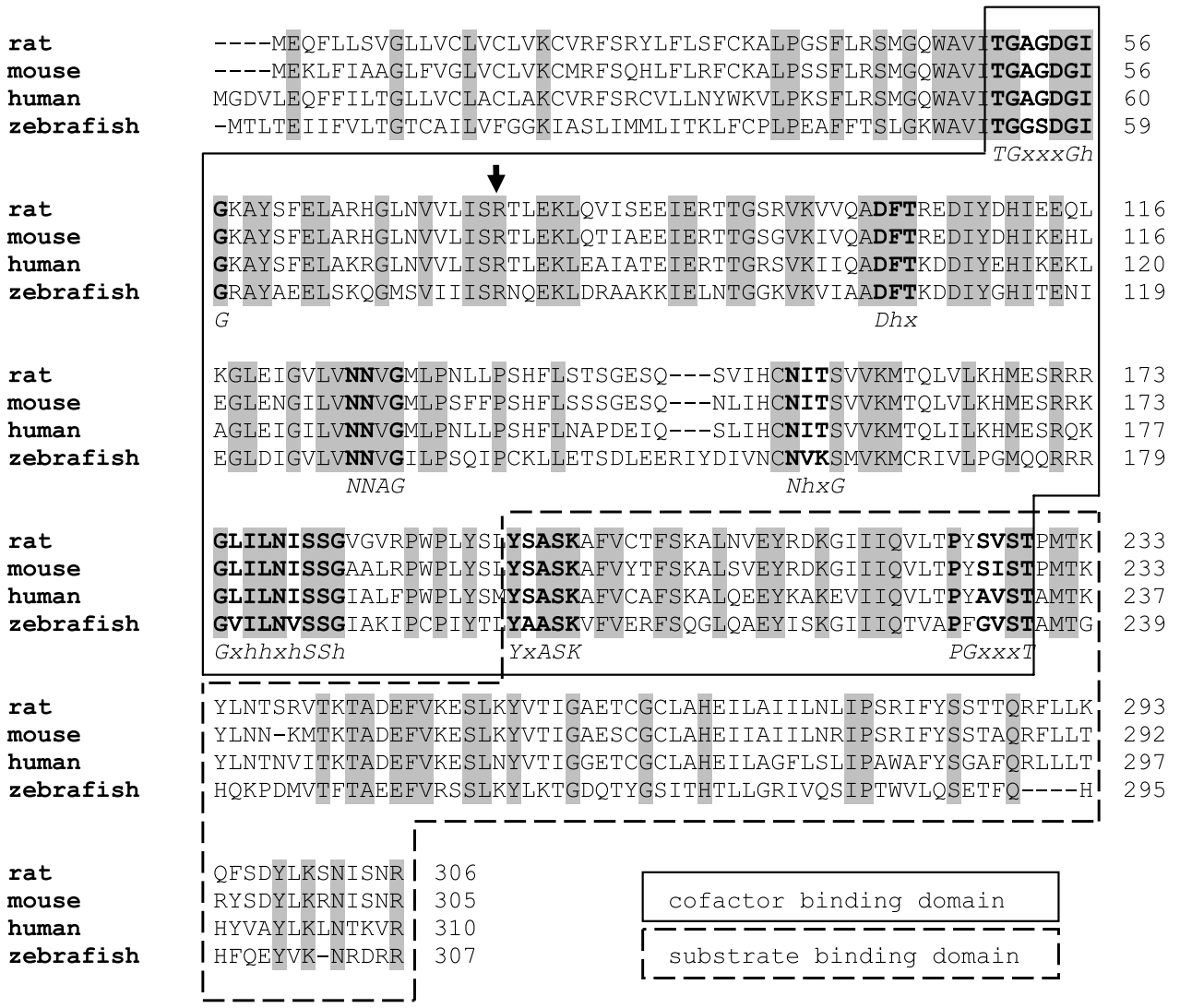


Figure 2 Alignment of the zebrafish and mammalian 17β-HSD 3 proteins. Typical features common to all four proteins are highlighted: functional motifs of the SDR family are indicated in bold with the consensus sequences written underneath. The amino acid R^{80} of the human enzyme, which conveys NADP(H) preference, is marked by an arrow. Domains predominantly involved in cofactor binding (including the SDR motifs) or substrate binding (from the catalytic site to the C-terminus) are boxed as indicated. Identical amino acids are on a gray background; h, hydrophobic residue; x, any aminoacid.

important motifs of the SDR family, to which 17β-HSD type 3 belongs, to be very well conserved. These motifs mediate cofactor binding (GxxxGhG), the catalytic mechanism (GxhhxhSSh and YxASK) and structural integrity (NNAG; Jornvall *et al.* 1995, Kallberg *et al.* 2002). Furthermore, residue R^{80}, which in the human 17β-HSD 3 determines the preference of NADPH over NADH (Geissler *et al.* 1994, McKeever *et al.* 2002), is conserved in all four enzymes. Overall amino acid identity is not the same between functionally different parts of the protein. In the preferentially cofactor-binding domain, encompassed by the SDR motifs, about 47% of residues are identical compared with 38% in the part exerting substrate-binding specificity (Fig. 2).

Zebrafish 17β-HSD 3 reduces androstenedione to testosterone with preference for NADPH as a cofactor

We tested whether zebrafish 17β-HSD 3 catalyzes the reduction of androstenedione to testosterone by use of the cofactor NADPH in transiently transfected cells. The zebrafish enzyme indeed catalyzed this reaction with high performance similar to human 17β-HSD 3 (Fig. 3a). There was a distinct and exclusive preference

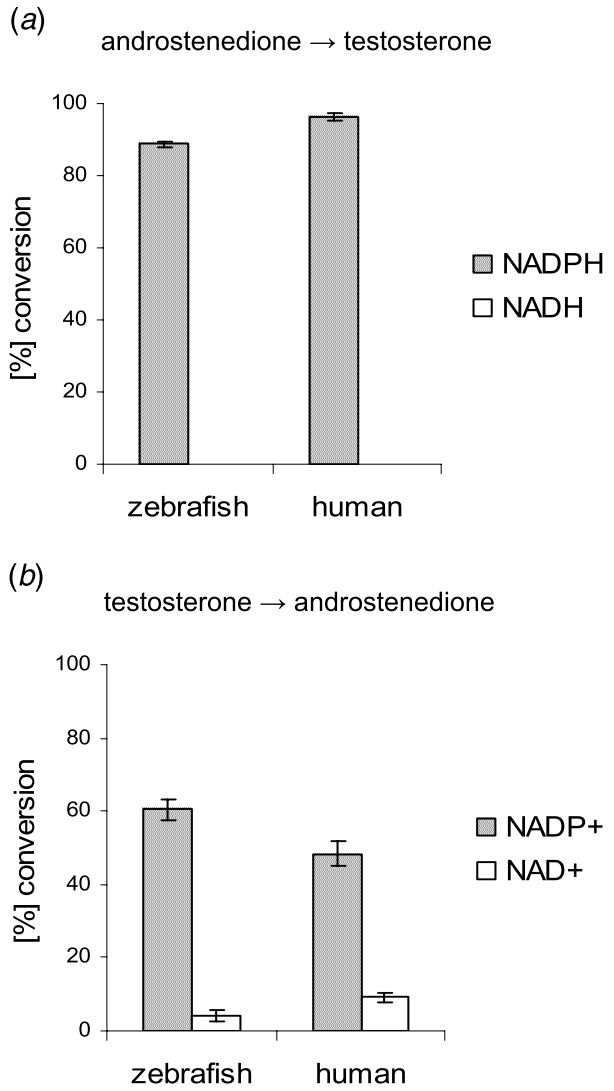


Figure 3 Cofactor preference and reaction direction of human and zebrafish 17β-HSD 3. The conversion of tritiated substrates was measured in zf4 cells transiently transfected with the zebrafish 17β-HSD 3 and HEK-293-Ebna cells transiently transfected with the human 17β-HSD 3. The potential of these cells to reduce androstenedione to testosterone (a) or to catalyze the reverse reaction (b) was investigated in the context of cofactor preference. Background activity of cells transfected with the empty vector was subtracted.

for NADPH over NADH as cofactor since with the latter no reduction could be detected. HEK-293 and zf4 cells transfected with the vector alone did not show any detectable reductive activity towards androstenedione (data not shown). The reverse reaction was also performed by both enzymes (Fig. 3b) but with an apparently lower efficiency compared with the reduction. Concerning the oxidation, even NAD^+ was accepted to some degree, giving rise to 5 and 9% product formation in case of zebrafish and human 17β-HSD 3, respectively. HEK-293 but not zf4 cells had some weak background activity in the oxidation of testosterone to androstenedione (data not shown), which was considered in data evaluation.

Zebrafish and human 17β-HSD 3 accept different androgens as substrates

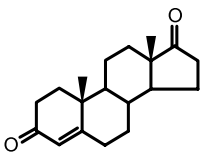
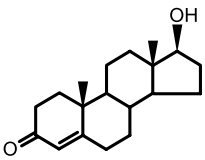
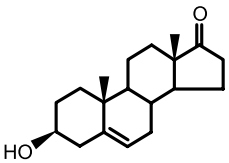
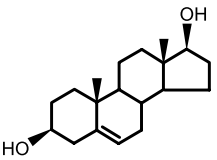
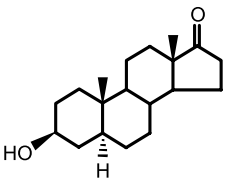
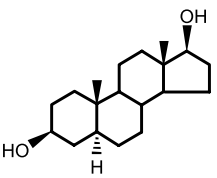
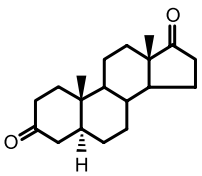
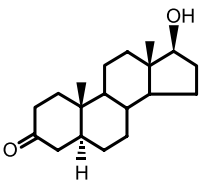
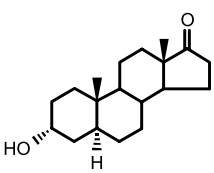
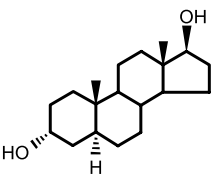
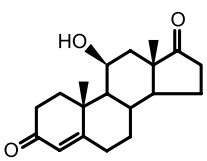
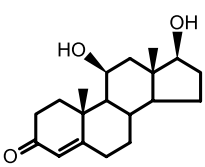
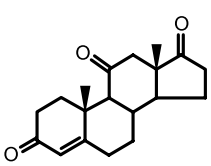
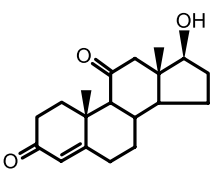
After measuring a high catalytic activity of zebrafish 17β-HSD 3 towards the reduction of androstenedione to testosterone we investigated whether other androgenic compounds are accepted as substrates and reduced at C-17. Since these other steroids were not available in tritiated form, the assays were performed with non-radioactive substrates and TLC. The results of these experiments are summarized in Table 1.

Reduction of androstenedione to testosterone was easily detectable with the less sensitive TLC method. We then tested further androgens occurring in the mammalian organism. Reduction of dehydroepiandrosterone (DHEA) at C-17 leads to 5δ-androstene-3β,17β-diol and this reaction was catalyzed by both human and zebrafish 17β-HSD 3, although the first seemed to be more effective (data not shown). Among the tested 5α-reduced androgens, only epiandrosterone was a substrate of both enzymes. Androsterone and androstanedione, though, were reduced at C-17 only by the human but not the zebrafish 17β-HSD 3. Since it is widely accepted that in fish 11-oxygenated androgens play a more important role than, for example, androstenedione, testosterone and dihydrotestosterone (DHT; Kime 1993, Redding & Patino 1993), we tested whether this type of androgen is a substrate for zebrafish 17β-HSD 3. Interestingly, 11-ketoandrostenedione as well as 11β-hydroxyandrostenedione was reduced at C-17, not only by the zebrafish but also by the human enzyme.

Zebrafish 17β-HSD 3 is widely expressed in the adult fish and during embryogenesis

17β-HSD 3 expression in tissues of male and female adults was investigated by RT-PCR and showed strong sexual dimorphism (Fig. 4a). Transcripts were detected in testis but surprisingly appeared to be present in a much higher degree in ovaries. In male fish the strongest signals were derived from liver. Furthermore, expression was readily detected in male spleen, kidney, heart and intestine whereas in the corresponding female tissues expression was hardly detectable or even absent. The enzyme might also display a sexually dimorphic expression in the skin for only extremely weak signals were obtained from this tissue from males compared with females. 17β-HSD 3 transcripts could also be

Table 1 Substrates tested for stereospecific reduction at position C-17 by zebrafish and human 17β-HSD 3

Reaction		Catalyzed by zebrafish 17β-HSD 3	Human 17β-HSD 3
Substrate	Product		
			
Androstenedione	Testosterone	Yes	Yes
			
Dehydroepiandrosterone	Androstene-3β,17β-diol	Yes	Yes
			
Epiandrosterone	Androstane-3β,17β-diol	Yes	Yes
			
Androstanedione	Dihydrotestosterone	No	Yes
			
Androsterone	Androstane-3β,17β-diol	No	Yes
			
11β-Hydroxy-androstenedione	11β-Hydroxy-testosterone	Yes	Yes
			
11-Ketoandrosterone	11-Ketotestosterone	Yes	Yes

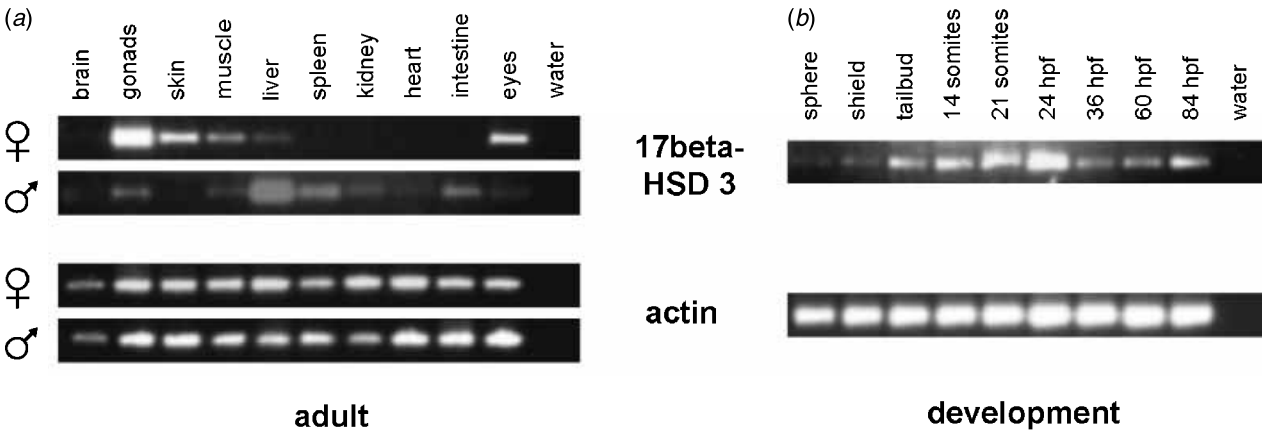


Figure 4 Expression pattern analysis of zebrafish 17β-HSD 3 in male and female adult fish and during embryonic development. Gel photographs show the observed distribution of 17β-HSD 3 transcripts as detected by RT-PCR in tissues of the adult fish (a) and during embryogenesis (b). Actin controls were included to ensure RNA quality and signal reproducibility. hpf, hours post-fertilization.

detected in muscle, eyes and, to a much lower degree, in the brain of both sexes.

During embryogenesis, 17β-HSD 3 mRNA was present from the sphere stage until hatching (Fig. 4b). The relative amounts, though, might vary between the different developmental stages since observed RT-PCR signals were strongest at the end of somitogenesis (21 somites, 24 h post-fertilization).

Zebrafish 17β-HSD 3 localizes to the ER

We monitored subcellular expression via detection of fluorescent proteins fused to the human and zebrafish enzymes transiently expressed in HeLa and zf4 cells, respectively (Fig. 5). 17β-HSD 3 tagged at the C-terminus showed a network-like expression centered around and extending from the nucleus which was reminiscent to the structure of the ER (Fig. 5a and c). In case of N-terminally tagged 17β-HSD 3 (Fig. 5b and d), localization was much more diffuse, reaching further into the cytoplasm but not fully occupying the cytoplasmic area. This localization pattern was distinctly different from that produced by C-terminally tagged proteins and as well from the localization of the fluorescent protein alone, which was equally distributed in the cytoplasm and nucleus (data not shown). Staining of the ER in HeLa cells (Fig. 5 g and h) and zf4 cells (Fig. 5e and f) by ER-Tracker dye revealed patterns similar to those of the C-terminally tagged proteins and produced a complete overlap with the latter expression pattern (Fig. 5i and k). Concerning N-terminally tagged 17β-HSD 3 though, the staining was rather dissimilar to that of the ER (Fig. 5j and l). Only very small overlaps were detected close to the nucleus in case of the zebrafish enzyme.

Discussion

17β-HSD 3 in an evolutionary context

The function of testosterone has been investigated in many vertebrates but apart from mammals only little is known about the molecular machinery controlling the formation of this steroid. 17β-HSD 3 has been suggested to be the major enzyme catalyzing the reduction of androstenedione to form testosterone and resulting in pseudohermaphroditism upon loss-of-function mutations in humans (Geissler *et al.* 1994). No non-mammalian 17β-HSD 3 has been characterized so far and we investigated whether the zebrafish homolog might play a similar role to that in mammals.

Screening the EST database at NCBI extracted several putative 17β-HSD 3 candidates and led to the identification of a single zebrafish homolog that shared about 45% amino acid identity with the mammalian enzymes. Phylogenetic analyses indicate that 17β-HSD type 3 is closely related to 17β-HSD type 12 and to 3-ketoacyl reductases. 17β-HSD type 12 is indeed the human homolog of yeast YBR159p, a 3-ketoacyl-CoA reductase involved in the elongation reaction to generate very-long-chain fatty acids (Moon & Horton 2003). It is as yet unknown whether 17β-HSD 12 also accepts steroids as substrates. Although its substrate specificity is different, 17β-HSD 3 still shares some typical aspects with its fatty acid-metabolizing relative. Elongation of very-long-chain fatty acids, for example, takes place at the ER membrane and, concordantly, human 17β-HSD 12 and the yeast homolog localize to this compartment via an N-terminal localization signal and a subsequent transmembrane helix (Han *et al.* 2002, Moon & Horton 2003). We found this to be true as well for zebrafish 17β-HSD 3, where a stretch of about 50 amino acids

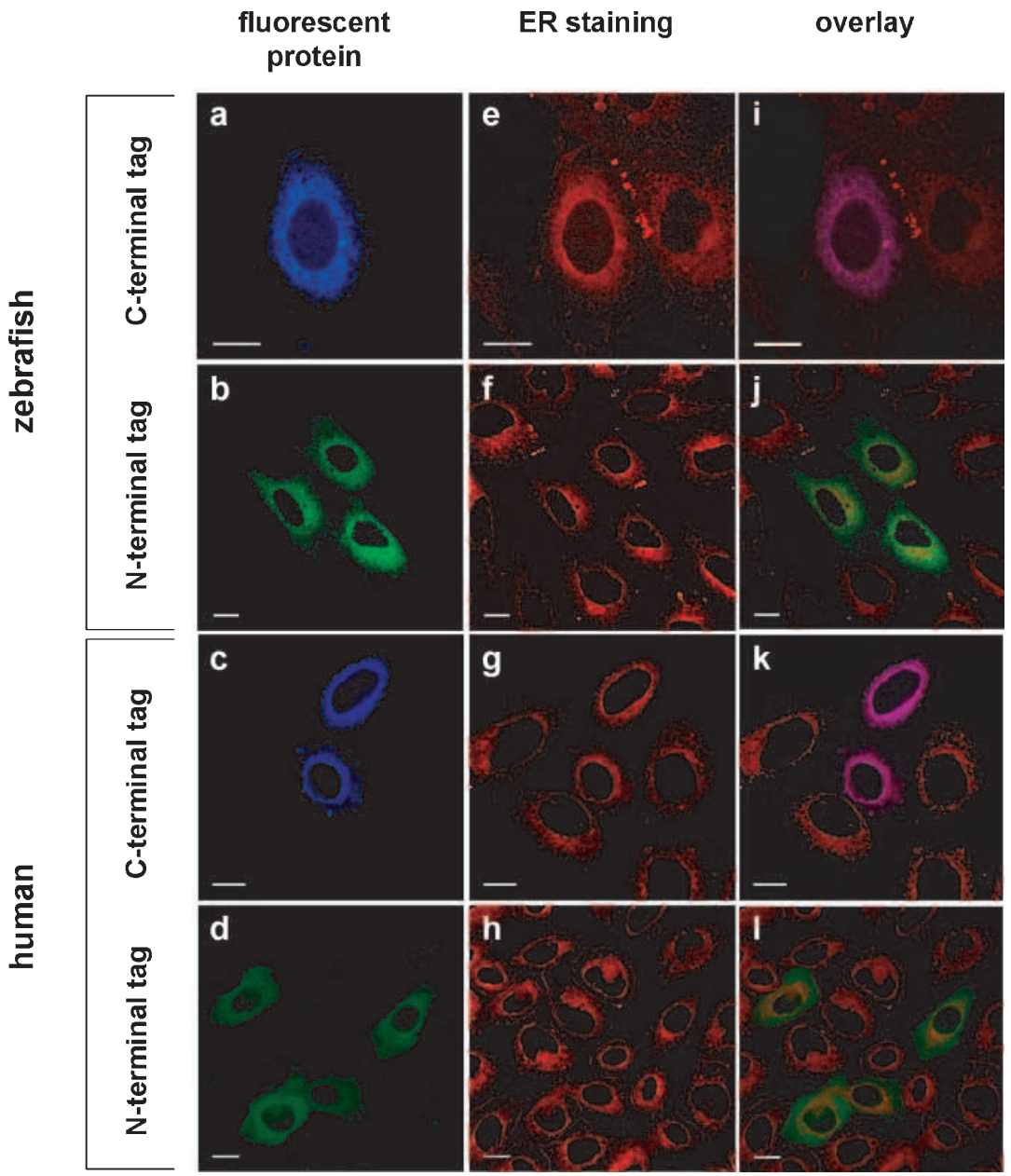


Figure 5 Subcellular localization of human and zebrafish 17β-HSD 3. Localization was studied in HeLa and zf4 cells transiently transfected with the human and zebrafish enzymes tagged with a fluorescent protein, respectively. Fluorescence of N-terminal tags with green fluorescent protein (b, d) is shown in green; C-terminal tags with cyan fluorescent protein (a) and yellow fluorescent protein (c) are pseudocolored in blue; (e–h) ER staining with ER Tracker (red); (i–l) overlay of ER staining and fluorescent protein signal. Overlay produces magenta staining in the case of C-terminal tags and yellow in the case of the N-terminal tag. Scale bar, 10 μm.

preceding the presumed start of the cofactor-binding site may facilitate the direction to and anchoring in the ER membrane. The presence of an N-terminal localization signal might also explain why addition of fluorescent protein to the C-terminus lead to localization at the ER membrane, whereas in the case of the N-terminal tag the putative localization signal seems to have been masked, leading to predominant localization to the cytosol and not the ER. The widespread expression of 17β-HSD 3, as observed in case of zebrafish, may also be inherited from the ancestor 17β-HSD 12, which is expressed ubiquitously (Moon & Horton 2003). Although human

17β-HSD 3 expression is often described as testis-specific, mRNA or protein have also been detected in adipose tissue (Corbould *et al.* 2002), brain (Stoffel-Wagner *et al.* 1999, Beyenburg *et al.* 2000) and bone (Feix *et al.* 2001). The murine homolog is expressed in a variety of male and female tissues (Sha *et al.* 1997).

Aside from 17β-HSD 3, other types of 17β-HSD are also able to catalyze the reduction of androstenedione to testosterone. In human, 17β-HSD 5 is widely expressed (Lin *et al.* 1997, Dufort *et al.* 1999, Qin & Rosenfield 2000) and accepts similar substrates as 17β-HSD 3. In mouse, 17β-HSD 5 is predominantly present in liver (Rheault *et al.* 1999) but murine 17β-HSD 1 has been demonstrated to convert androstenedione to testosterone with similar efficiency to 17β-HSD 3 (Nokelainen *et al.* 1996) and is expressed in many different organs (Sha *et al.* 1997). The ability to form testosterone by reduction of androstenedione might be present almost ubiquitously in the vertebrate organism, as was also suggested by measurements of androgenic activity in several tissues of rat (Martel *et al.* 1992) and monkey (Labrie *et al.* 1997). On the molecular level though, employment of 17β-HSD 3 in this context might have evolved differently and be species-specific.

In zebrafish at least, widespread expression of 17β-HSD 3, for example during embryogenesis and in male adults, may also indirectly serve in the formation of estradiol. Here, 17β-HSD 1 was found not to be responsible for provision of estradiol, as it could not be detected in the respective tissues (Mindnich *et al.* 2004). Since aromatase is present during embryogenesis (Kishida & Callard 2001, Trant *et al.* 2001) and in the male zebrafish (Goto-Kazeto *et al.* 2004), estradiol could be formed via aromatization of testosterone generated from androstenedione by reductive activity of 17β-HSD 3.

Enzymatic properties of human and zebrafish 17β-HSD 3

In our experiments, several structurally different androgens, such as DHEA, epiandrosterone and 11-ketotestosterone, were substrates of human and zebrafish 17β-HSD 3. But concerning 5α-reduced compounds there was a marked difference between these two enzymes since only the human protein was able to reduce androsterone and androstanedione. Comparison of the three-dimensional structure of all measured substrates shows that, in all cases, where the zebrafish 17β-HSD 3 accepts and converts the steroid, this keto- or hydroxy-group at C-3 is in or above the plane formed by the A-ring, as can be seen in androstenedione, DHEA and epiandrosterone. In androstanedione and androsterone though, the keto- and hydroxy-group are below this plane. Unfortunately, no crystal structure of 17β-HSD 3 is so far available through which this theory of stereo-specific interaction with the C-3 oxy-group can be investigated further. Taking into account the general architecture of SDRs the most C-terminal part of the enzyme likely conveys recognition and stabilization of this part of the steroid. In this region, zebrafish 17β-HSD 3 lacks at least four amino acids, including an arginine, conserved in the mammalian homologs (see also Fig. 2). This deviation may result in a structural difference and subsequent loss of stabilization of steroids where the oxy-group at C-3 is oriented below the steroid plane. That certain DHT derivatives are not substrates of zebrafish 17β-HSD 3 may be because they do not have any important functions in fish compared with mammals. It has been generally accepted that, instead of DHT, 11-ketotestosterone is biologically the most important androgen in teleosts (Kime 1993), which was supported by the findings of Miura *et al.* (1991) that 11-ketotestosterone by itself induced complete spermatogenesis in the Japanese eel. DHT has been detected in several teleost species (Kime 1993) and teleost androgen receptors may sometimes display an affinity to 5α-reduced androgens similar to or even greater than to 11-oxygenated androgens (Ikeuchi *et al.* 1999, Sperry & Thomas 2000, Braun & Thomas 2004). In the case of zebrafish, there are no data available on the true molecular character of the biologically active androgens and the androgen receptor itself has not been characterized so far. Our finding that at least some 5α-reduced androgens are no longer substrates of 17β-HSD 3 in zebrafish, suggest these androgens to be of minor importance for this organism or, alternatively, that this specific enzymatic function has been taken over by another, as-yet unidentified, 17β-HSD.

Surprisingly, some androgens that might be considered fish-specific, such as 11β-hydroxyandrostenedione and 11-ketoandrostenedione, we found to be substrates not only of zebrafish but also of human 17β-HSD 3. In fish, this may contribute to, but not directly lead to, the formation of 11-ketotestosterone, which is considered to be formed by 11β-hydroxysteroid dehydrogenase (11β-HSD) from 11β-hydroxytestosterone (Kusakabe *et al.* 2003). 11-Oxygenated androgens are also present in mammals (Oertel & Eik-Nes 1962, Kecskes *et al.* 1982) due to the activity of 11β-hydroxylase. Classically, this enzyme is part of the corticoid metabolism leading to the formation of corticosterone and cortisol. 11β-HSD catalyzes the interconversion of cortisol and cortisone, regulating glucocorticoid activity. 11β-Hydroxytestosterone and 11-ketotestosterone can inhibit the oxidative and reductive functions of 11β-HSD, respectively, and thereby may influence the local formation of glucocorticoids (Wang *et al.* 2002). Both modified androgens are significantly stronger inhibitors than testosterone (Wang *et al.* 2002), so that production of these steroids by 17β-HSD 3 may be of physiological importance in the crosstalk between glucocorticoid and androgen metabolism.

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