Sulphonation of dehydroepiandrosterone and neurosteroids: molecular cloning, expression, and functional characterization of a novel zebrafish SULT2 cytosolic sulphotransferase

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By searching the zebrafish EST (expressed-sequence tag) database, we have identified two partial cDNA clones encoding the 5' and 3' regions of a putative zebrafish sulphotransferase (ST). Using the reverse transcription-PCR technique, a full-length cDNA encoding this zebrafish ST was successfully cloned. Sequence analysis revealed that this novel zebrafish ST displays 44 %, 43 % and 40 % amino acid identity with mouse SULT2B1, human SULT2B1b and human SULT2A1 ST respectively. This zebrafish ST therefore appears to belong to the SULT2 cytosolic ST gene family. Recombinant zebrafish ST, expressed using the pGEX-2TK prokaryotic expression system and purified from transformed *Escherichia coli* cells, migrated as a 34 kDa protein upon SDS/PAGE. Purified zebrafish ST displayed a strong sulphonating activity toward DHEA (dehydroepiandrosterone), with an optimum

pH of 9.5. The enzyme also exhibited activities toward several neurosteroids with differential K_m and V_{max} values. A thermostability experiment revealed the enzyme to be relatively stable over a temperature range between 20 °C and 43 °C. Among ten different divalent metal cations tested, Fe^{2+} and Cd^{2+} exhibited small, but significant, stimulatory effects, whereas Hg^{2+} and Cu^{2+} displayed considerably stronger inhibitory effects on the DHEA-sulphonating activity of the enzyme. These results constitute the first study on the molecular cloning, expression, and characterization of a zebrafish cytosolic SULT2 ST.

Key words: dehydroepiandrosterone (DHEA), molecular cloning, neurosteroid, sulphotransferase, SULT2, zebrafish.

INTRODUCTION

Cytosolic sulphotransferases (STs) in mammals are generally known as Phase II drug-metabolizing/detoxifying enzymes that catalyse the sulphonation of a variety of compounds containing hydroxy or amino groups, using PAPS (3'-phosphoadenosine 5'-phosphosulphate) as the sulphonyl group donor [1–3]. Increasingly, they have been shown to also be involved in the regulation of the levels and activities of endogenous molecules such as thyroid and steroid hormones, catecholamine hormones/neurotransmitters, and bile acids [4,5]. A prominent example among this latter group of compounds is DHEA (dehydroepiandrosterone). In humans, DHEA is the principal C_{19} steroid produced by the adrenals. The bulk of DHEA produced is sulphated and secreted into circulation, which then serves as a precursor for the synthesis of androgenic and oestrogenic steroids in extra-adrenal tissues [6,7]. It has also been shown that unconjugated and sulphate-conjugated DHEA (DHEA-S) are present in mammalian brain [8–10]. Therefore DHEA and its biosynthetic precursor, pregnenolone, and some biologically active metabolites, including DHEA-S, have been collectively called the neurosteroids [8,11]. Both DHEA and DHEA-S have been suggested to provide some protective effects against cardiovascular disease, obesity, hypercholesterolaemia, cancer, Alzheimer's disease, insulin-dependent diabetes mellitus and other immune-modulated diseases [12–15].

Based on the amino acid sequences of known mammalian cytosolic STs, several gene families have been categorized within the cytosolic ST gene superfamily. Two major gene families among them are the PST (phenol ST) family (designated SULT1) and the HSST (hydroxysteroid ST) family (designated SULT2) [16,17]. The PST family consists of at least four subfamilies, PSTs (SULT1A), dopa/tyrosine (or thyroid hormone) STs (SULT1B), hydroxyarylamine (or acetaminofluorene) STs (SULT1C) and oestrogen STs (SULT1E). The HSST family presently comprises two subfamilies, DHEA STs (SULT2A) and cholesterol STs (SULT2B). Many cytosolic STs have been rather well-characterized in terms of their biochemical properties and structure–function relationships. Relatively little, however, is known with regard to the regulation of the expression and activity of cytosolic STs. Some studies have shown that certain divalent metal cations may exert stimulatory or inhibitory effects on the activities of cytosolic STs [18,19]. Using mouse or rat as the model, the expression of some cytosolic STs has been demonstrated to be age- or sex-dependent [20,21]. The implications of this information in the context of physiology or toxicology, however, remain to be clarified.

Zebrafish has, in recent years, emerged as a popular animal model for a wide range of studies [22,23]. Its advantages, compared with mouse, rat, or other vertebrate animal models, include the small size (3–4 cm in length for adult fish), availability of relatively large number of eggs (> 200 eggs/week per mature

Abbreviations used: Caps, 2-(N-cyclohexylamino)-1-propanesulphonic acid; Ches, 2-(N-cyclohexylamino)ethane sulphonic acid; DHEA, dehydroepiandrosterone; DHEA-S, sulphate-conjugated DHEA; EST, expressed-sequence tag; GST, glutathione S-transferase; HSST, hydroxysteroid ST; IPTG, isopropyl β -D-thiogalactoside; PAPS, 3'-phosphoadenosine 5'-phosphosulphate; PST, phenol ST; RT-PCR, reverse transcription-PCR; ST, sulphotransferase; T_3 , 3,3',5-tri-iodo-L-thyronine.

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The nucleotide sequence data reported for zebrafish sulphotransferase has been submitted to the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AY181063.

female), rapid external development of virtually transparent embryos (in 2–4 days) and short generation time (approx. 3 months). These unique characteristics of the zebrafish make it an excellent model for a systematic investigation on the ontogeny of the expression of individual cytosolic STs and their tissue- and cell-type-specific distribution, as well as the physiological relevance of individual cytosolic STs. A prerequisite for using zebrafish in these studies, however, is the identification of the various cytosolic STs and their biochemical characterization.

The present study is the first study on the molecular cloning of a novel zebrafish SULT2 cytosolic ST. The enzyme was expressed in recombinant form and its enzymic activities toward various endogenous compounds, in particular DHEA and other neurosteroids, as well as some xenobiotics, were examined.

EXPERIMENTAL

Materials

p-Nitrophenol, dopamine, 2-naphthol, 2-naphthylamine, aprotinin, thrombin, 3,3',5-tri-iodo-L-thyronine (T_3 , sodium salt), thyroxine, oestrone [1,3,5(10)-oestratrien-3-ol-17-one], DHEA, ATP, SDS, Hepes, Taps, Trizma base, DTT (dithiothreitol) and IPTG (isopropyl β -D-thiogalactoside) were products from Sigma Chemical Company. Pregnenolone, 17 α -hydroxypregnenolone, progesterone, 17 α -progesterone, androstenedione, allopregnanolone, corticosterone, cortisol and carrier-free $Na_2^{35}SO_4$ were from ICN Biomedicals (Costa Mesa, CA, U.S.A.). AmpliTaq DNA polymerase was a product of PerkinElmer. Takara *Ex Taq* DNA polymerase was purchased from PanVera Corporation (Madison, WI, U.S.A.). T4 DNA ligase and all restriction endonucleases were from New England Biolabs. XL1-Blue MRF' and BL21 *Escherichia coli* host strains were purchased from Stratagene. Oligonucleotide primers were synthesized by MWG Biotech (High Point, NC, U.S.A.). pGEX-2TK GST (glutathione S-transferase) gene fusion vector, glutathione-Sepharose 4B and First Strand cDNA Synthesis Kit were from Amersham Biosciences. Recombinant human bifunctional ATP sulphurylase/adenosine 5'-phosphosulphate kinase was prepared as described previously [24]. TRI Reagent® was from Molecular Research Center (Cincinnati, OH, U.S.A.). Total RNA from zebrafish was prepared using the TRI Reagent® according to the manufacturer's instructions. Cellulose TLC plates were products of EM Science (Darmstadt, Germany). All other reagents were of the highest grades commercially available.

Molecular cloning of a novel zebrafish cytosolic ST

By searching the EST (expressed-sequence tag) database, two zebrafish cDNA clones (GenBank® accession number BI562822 and BG985485) encoding, respectively, 5' and 3' regions of a putative cytosolic ST were identified. Reverse transcription-PCR (RT-PCR) was employed to amplify this novel zebrafish ST nucleotide sequence. With the zebrafish total RNA as template and oligo(dT) as the primer, the first strand cDNA was synthesized using the First Strand cDNA Synthesis Kit. Using sense (5'-CGCGGATCCATGACGGAAGCAGAGCTGTATTCCTC-3') and antisense (5'-CGCGGATCCCTCAGTCCCATGGAAAGCTGAACTTCAC-3') oligonucleotide primers (designed based on 5'- and 3'-coding regions reported for the two above-mentioned zebrafish ST cDNA clones and with *Bam*HI restriction site incorporated at the end), PCR in a 100 μ l reaction mixture was carried out under the action of *Ex Taq* DNA polymerase, with zebrafish first-strand cDNA prepared as template. Amplification conditions were 25 cycles of 1 min at 94 °C, 1 min at 60 °C and 1 min at

72 °C. The final reaction mixture was applied on to a 1.2 % (w/v) agarose gel, separated by electrophoresis, and visualized by ethidium bromide staining. The PCR product detected was excised from the gel, and the DNA fragment therein was isolated by spin filtration. Purified PCR product was restricted with *Bam*HI restriction endonuclease, subcloned into the *Bam*HI site of pGEX-2TK, and subjected to nucleotide sequencing using, respectively, GEX-5' (5'-GGGCTGGCAAGCCACGTTTGGTG-3') and GEX-3' (5'-CCGGGAGCTGCATGTGTTCAGAGG-3') as primers.

Bacterial expression and purification of the recombinant zebrafish cytosolic ST

pGEX-2TK harbouring the cloned zebrafish ST cDNA was transformed into competent *E. coli* BL21 cells. Transformed BL21 cells were grown to a D_{600} of approx. 0.5 in 1 litre of LB (Luria–Bertani) medium supplemented with 100 μ g/ml ampicillin, and induced with 0.1 mM IPTG. After an overnight induction at room temperature (22 °C), the cells were collected by centrifugation at 1500 *g* for 30 min at 4 °C and were homogenized in 20 ml of ice-cold lysis buffer (10 mM Tris/HCl, pH 8.0, 150 mM NaCl and 1 mM EDTA) using an Aminco French Press. The protease inhibitor aprotinin (20 μ l at 10 mg/ml) was added to the crude homogenate before centrifugation at 10 000 *g* for 30 min at 4 °C. The supernatant collected was fractionated using 0.5 ml of glutathione-Sepharose, and the bound GST fusion protein was treated with 2 ml of a thrombin digestion buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl and 2.5 mM $CaCl_2$) containing 5 units/ml bovine thrombin. Following 30 min of incubation at room temperature with constant agitation, the preparation was subjected to centrifugation at 1500 *g* for 30 min at 22 °C. The recombinant zebrafish ST present in the collected supernatant was analysed with respect to its enzymic properties.

Enzymic assay

The ST activities were assayed using [35 S]PAPS as the sulphonyl group donor. The standard assay mixture, with a final volume of 25 μ l, contained 50 mM potassium phosphate buffer (pH 7.0), 14 μ M [35 S]PAPS (15 Ci/mmol) and 50 μ M substrate. The reaction was started by the addition of the enzyme (0.025 μ g/25 μ l of assay mixture), allowed to proceed for 3 min at 28 °C, and terminated by heating at 100 °C for 2 min. The precipitates formed were cleared by centrifugation at 13 000 *g* for 2 min at 22 °C, and the supernatant was subjected to the analysis of [35 S]sulphated product using the previously developed TLC separation procedure [25], with *n*-butanol/isopropanol/88 % formic acid/water (2:1:1:2; by volume) as the solvent system. To examine the pH-dependence of the DHEA-sulphonating activity, different buffers {50 mM Mes at pH 5.5 or 6.0, Mops at pH 6.5 or 7.0, Taps at pH 7.5, 8.0 8.5 or 9.0, Ches [2-(*N*-cyclohexylamino)ethanesulphonic acid] at pH 9.0 or 9.5, and Caps [3-(cyclohexylamino)-1-propanesulphonic acid] at pH 9.5, 10.0, 10.5 or 11.0} instead of 50 mM potassium phosphate buffer (pH 7.0) were used in the reactions. For the kinetic studies on the sulphonation of DHEA or other neurosteroids, various concentrations (ranging from 1 to 500 μ M) of these latter substrate compounds and 50 mM Ches at pH 9.5 were used. SigmaPlot 2001 for Windows (version 7.0) was used for the non-linear regression analysis of the kinetic data. The equation used for fitting the data was $v = V_{max}/(1 + K_m/[S] + [S]/K_i)$, which was the derivation of Michaelis–Menten equation for substrate inhibition. K_i represents the dissociation constant for the inhibitory SES (substrate–enzyme–substrate) ternary complex. To evaluate its thermostability, the zebrafish ST was first incubated

for 15 min at, respectively, 20 °C, 28 °C, 37 °C, 43 °C and 48 °C, and then assayed for its DHEA-sulphonating activity at 28 °C. To determine the stimulatory/inhibitory effects of divalent metal cations on the zebrafish ST, enzymic assays in the presence or absence of divalent metal cations were performed as described above.

Miscellaneous methods

[³⁵S]PAPS (carrier-free) was synthesized from ATP and carrier-free Na₂³⁵SO₄ using the human bifunctional ATP sulphurylase/APS (adenosine 5'-phosphosulphate) kinase as described previously [26]. The synthesized [³⁵S]PAPS was then adjusted to the required concentration and specific radioactivity by the addition of unlabelled PAPS. The concentration of PAPS was confirmed by measuring its absorbance at 260 nm [27]. SDS/PAGE was performed on a 12 % (w/v) polyacrylamide gel using the method of Laemmli [28]. Protein determination was based on the method of Bradford with bovine serum albumin as the standard [29].

RESULTS AND DISCUSSION

Despite the considerable progress made in recent years on the cytosolic STs, several fundamental issues concerning their ontogeny, regulation and physiological involvement still remain to be fully elucidated. The present study was prompted by an attempt to develop a zebrafish model in order to address these important issues. As a first step toward achieving this goal, we have started investigating the various cytosolic STs that are present in zebrafish. By searching the sequences stored in the zebrafish EST database, we have identified two partial cDNA clones encoding a putative zebrafish ST. Indeed, employing the RT-PCR technique, a full-length cDNA encoding the zebrafish ST was successfully cloned.

Molecular cloning of the zebrafish cytosolic ST

The RT-PCR-cloned zebrafish ST cDNA was subjected to nucleotide sequencing in both directions. The nucleotide sequence obtained has been submitted to the GenBank® database under the Accession number AY181063. Figure 1 shows the nucleotide and deduced amino acid sequences of this zebrafish ST. Similar to known mammalian cytosolic STs, this zebrafish ST contains sequences (as underlined in Figure 1) resembling the so-called 'signature sequences' (YPSKSGTXW in the N-terminal region and RKGXXGDWKNXFT in the C-terminal region) [17] that have been proposed to be involved in the binding of PAPS, a co-substrate for the ST-catalysed sulphonation reactions [30]. Sequence analysis based on BLAST search revealed that the deduced amino acid sequence of the zebrafish ST displayed, respectively, 44 %, 43 % and 40 % identity with those of mouse SULT2B1, human SULT2B1b, and hSULT2A1 ST [17]. Lower amino acid identity values were found in comparison with other known mammalian cytosolic STs in SULT1 or other ST gene families. It is generally accepted that members of the same ST gene family share at least 40–45 % amino acid sequence identity, whereas members of subfamilies divided further in each ST gene family are > 60 % identical in amino acid sequence [16,17]. Based on these criteria, the new zebrafish ST, although belonging to the SULT2 gene family, cannot be classified into any of the existing subfamilies within SULT2. Figure 2 shows a phylogenetic tree constructed based on amino acid sequence alignment (with bootstrapping) of the zebrafish SULT2 ST and known human and mouse cytosolic STs, using the Gene Bee server at Moscow State University, Moscow, Russia.

10	20	30	40	50	60
ATGACGGAAGCAGAGCTGTATTCCTCTTATAAAGGTGTGTTAGTCCTTCATATGTACAC					
M T E A E L Y S S Y K G V L V P S Y V H					
70	80	90	100	110	120
CCTGCTGAGAGCCGTAATACTACGAGGACTTCACCTTCGCTCCGATGACATCCTGATC					
P A E S L K Y Y E D F T F R P D D I L I					
130	140	150	160	170	180
ATCACTTTCCCTAAATCAGGCACCATGGATGCAGGAGATCGTCCCTCGGTAGTGAGC					
I T F P K S G T T W M Q E I V P L V V S					
190	200	210	220	230	240
GAGGGCGATCTGACGCCGTTCTGACCGTGCCTCACTGGGACCGAGTGCCTGGTGGAG					
E G D L T P V L T V P N W D R V P W L E					
250	260	270	280	290	300
GAGCATCGAGCCATCTGCTGAGTTAGAGCAGAGCATCGCCACGCATCTTCGCCACT					
E H R A I L L S L E Q R A S P R I F A T					
310	320	330	340	350	360
CACCTTACCCAGATGATGAACCCGCTCTACTTCAAGATCAAGCCAGGGTCTGTAT					
H F H H Q M M N P S Y F K I K P R V L Y					
370	380	390	400	410	420
GTCATGAGGAACCCCAAGACGCTCTTCATTTCTTCATATCACTCCATGAGATGGCCTCG					
V M R N P K D V F I S S Y H F H E M A S					
430	440	450	460	470	480
TTCTTGCTGAGCCCGGGAACACAGACGAGTTTCATGAGAGAGTTCTCGATGGAACAATC					
F L V S P G T Q D E F M E K F L D G T I					
490	500	510	520	530	540
ATGTTTGCTCTGCTTGGACCATGTGAAAAGCTGGCTGACCGTGGAGAACAGGAGCAC					
M F G S W F D H V K S W L N A G E Q E H					
550	560	570	580	590	600
ATCCTCTACCTCTGCTACGAGGACATGATCTCTGATCTGAAAGCCTCTGTTGGAGAAGATC					
I L Y L C Y E D M I S D L K A S V E K I					
610	620	630	640	650	660
GCCACTTTTCTGGGAAATCCTTGAGCTCAGAGGTGGTGGAGAAATCGCAGATCACTGT					
A T F L G K S L S S E V V E K I A D H C					
670	680	690	700	710	720
GTGTTGAGAAACATGAAGCAGAACAGATGTCCAACTACTCTCTGTTGCCGAGGAGTTC					
V F E N M K Q N K M S N Y S L V P E E F					
730	740	750	760	770	780
ATGGACAGAGAAGTCAGAGTTTCTGAGGAAAGGAATTTGTCGATTTGGAAGAATCAT					
M D Q K S E F L R K G I V G D W K N H					
790	800	810	820	830	840
TTCAAGCATGCTCAAGAGCAGAAGTTTAATGCTGTTTATCAAGAGAAGATGAAGATGTG					
F S T A Q E Q K F N A V Y Q E K M K D V					
850	860	870			
AAGTTCACTTTCCATGGGACTGA					
K F S F P W D *					

Figure 1 Nucleotide and deduced amino acid sequences of the zebrafish ST cDNA

Nucleotides are numbered in the 5' to 3' direction, and the translation stop codon is indicated by an asterisk.

Expression, purification and characterization of recombinant zebrafish cytosolic ST

Recombinant zebrafish ST was expressed in BL21 *E. coli* cells using the pGEX-2TK GST gene fusion system. As shown in Figure 3, the recombinant zebrafish ST, cleaved from the glutathione–Sepharose-fractionated fusion protein by thrombin, migrated by SDS/PAGE to a position corresponding to a mass of approx. 34 kDa. These results are in agreement with the molecular mass (33 633 Da) calculated based on their deduced amino acid sequences. The purified recombinant zebrafish ST was subjected to functional characterization with respect to its enzymic activities. In the first set of experiments, a variety of endogenous or xenobiotic compounds were tested as substrates for the recombinant zebrafish ST. Activity data compiled in Table 1 revealed that, among the 27 compounds tested, the zebrafish ST displayed a strong activity toward DHEA, a much weaker activity toward T₃, and virtually no activities toward the rest of the compounds tested. The specificity of the enzyme for DHEA is in line with its being a member of the SULT2 gene family (cf. Figure 2). Using DHEA as the substrate, the pH-dependence of the purified zebrafish ST was examined. As shown in Figure 4, the enzyme exhibited a pH optimum at 9.5. This pH optimum is considerably different from those of human SULT2A1 (DHEA) ST (pH 7–9) [31], and human SULT2B1a (pregnenolone) and SULT2B1b (cholesterol) STs (pH 7.08 and 7.34 respectively) [32].

Figure 2 Classification of the zebrafish ST on the basis of deduced amino acid sequence

The dendrogram shows the degree of amino acid sequence homology among cytosolic STs. For references for individual STs, see the review by Weinshilboum et al. [17]. ZF, zebrafish; h, human; m, mouse.

Figure 3 SDS gel electrophoretic pattern of purified recombinant zebrafish ST

Purified zebrafish ST sample was subjected to SDS/PAGE on a 12% (w/v) polyacrylamide gel, followed by Coomassie Blue staining. Protein molecular-mass markers were co-electrophoresed in lane 2 and sizes are given in kDa to the right of the gel.

Since, as mentioned in the Introduction, DHEA has been suggested to function as a neurosteroid, we were interested in finding out whether other neurosteroids can also be used as substrates for the purified zebrafish ST. As shown in Table 2, among the nine neurosteroids tested, the enzyme showed activities, in decreasing order, toward only DHEA, pregnenolone, allopregnanolone and 4-androstene-3,17-dione. To investigate in more detail the sulphonation of these neurosteroids, the kinetics of sulphonation of these compounds by the zebrafish ST was examined. Data

Table 1 Specific activity of the zebrafish SULT2 ST toward endogenous and xenobiotic compounds

Specific activity refers to nmol substrate sulphated/min per mg of purified enzyme. Enzymic assays were performed at pH 7.0. Data represent means $\pm$ S.D. derived from three experiments.

Substrate	Specific activity (nmol/min per mg of enzyme)
DHEA	2.9 $\pm$ 0.1
T ₃	0.1 $\pm$ 0.1
Thyroxine (T ₄)	0
Oestrone	0
Dopamine	0
L-Dopa	0
D-Dopa	0
p-Nitrophenol	0
2-Naphthylamine	0
2-Naphthol	0
Butylated hydroxyanisole	0
Butylated hydroxytoluene	0
Daidzein	0
Kaempferol	0
Caffeic acid	0
Genistein	0
Myricetin	0
Quercetin	0
Gallic acid	0
Chlorogenic acid	0
Catechin hydrate	0
Epicatechin (EC)	0
Epigallocatechin gallate (EGCG)	0
n-Propyl gallate	0
Dexamethasone	0
Acetaminophen	0
Minoxidil	0

Figure 4 pH-dependency of the DHEA-sulphonating activity of purified zebrafish ST

The enzymic assays were carried out under standard assay conditions as described in the Experimental section using different buffer systems as indicated. The data represent calculated mean values derived from three experiments.

Table 2 Specific activity of the zebrafish SULT2 ST toward neurosteroids

Specific activity refers to nmol of substrate sulphated/min per mg of purified enzyme. Enzymic assays were performed at pH 9.5. Data represent means $\pm$ S.D. derived from three experiments.

Substrate	Specific activity (nmol/min per mg of enzyme)
DHEA	24.2 $\pm$ 0.5
Pregnenolone	19.6 $\pm$ 0.1
Allopregnanolone	6.6 $\pm$ 0.3
4-Androstene-3,17-dione	2.8 $\pm$ 0.1
17 α -Hydroxypregnenolone	1.1 $\pm$ 0.1
Hydrocortisone	0
Progesterone	0
17 α -Hydroxyprogesterone	0
Corticosterone	0

Table 3 Kinetic constants of zebrafish SULT2 ST with neurosteroids as substrates

Data shown represent means $\pm$ S.D. derived from three determinations.

Substrate	K_m (μ M)	V_{max} (nmol/min per mg)	K_i (μ M)
DHEA	134 $\pm$ 18	92 $\pm$ 8	1000 $\pm$ 270
Pregnenolone	44 $\pm$ 25	46 $\pm$ 15	350 $\pm$ 240
4-Androstene-3,17-dione	266 $\pm$ 23	18 $\pm$ 1	—*
Allopregnanolone	48 $\pm$ 13	15 $\pm$ 2	420 $\pm$ 140

* Substrate inhibition was not observed. K_i was approx. 3.7 M according to the non-linear regression analysis.

obtained were analysed using a non-linear regression program (SigmaPlot 2001). Table 3 shows the kinetic constants determined for the sulphonation of DHEA and the other three neurosteroids. Compared with the kinetic constants determined for the sulphonation of DHEA, cholesterol or pregnenolone by human SULT2A1 (DHEA) ST, SULT2B1a ST or SULT2B1b ST [31–33],

Figure 5 Stability of the zebrafish ST upon incubation at different temperatures

The figure shows the relative activity of purified zebrafish ST incubated for 15 min at different temperatures, followed by enzymic assay under standard conditions, as described in the Experimental section. The data represent calculated mean values derived from three experiments.

the zebrafish enzyme exhibited considerably higher K_m values and comparable V_{max} values. Like many mammalian cytosolic STs [1–5], substrate inhibition was observed for the zebrafish ST. Pregnenolone and allopregnanolone showed considerable substrate inhibition at about 100 μ M, whereas DHEA exhibited significant inhibition at 500 μ M. The K_i values determined were in the same range. The physiological relevance of the sulphonation of neurosteroids [34,35] by this novel zebrafish ST remains to be clarified. Elucidation of the tissue specificity of expression of this enzyme may throw some light on this problem. It is worthwhile pointing out that, using the rat as a model, neurosteroid sulphate levels have been shown to be ten times higher in brain than in serum [8], and a SULT2A1 ST exhibiting neurosteroid-sulphonating activities has been demonstrated to be expressed in rat brain [36]. Along the same line, there have been studies showing the existence of neurosteroids and neurosteroid-metabolizing enzymes in fish [37,38]. It should be remembered, however, that, in humans, DHEA-S has been shown to be generated and secreted by the adrenals [6,7], where DHEA (SULT2A1) ST is also expressed [39]. Therefore, a role of the zebrafish ST in the sulphonation of DHEA in non-nervous tissues can not be ruled out.

Zebrafish are normally maintained in aquaria heated to 28 °C [40]. In their natural habitat, however, they are subjected to fluctuation in body temperature. An intriguing issue, therefore, is related to its stability at different temperatures. A thermostability experiment was carried out in which the recombinant zebrafish ST was first incubated for 15 min at different temperatures, followed by enzymic assay under standard conditions (see the Experimental section) with DHEA as the substrate. As shown in Figure 5, activity data obtained indicated that the zebrafish ST was stable over a relatively wide range of temperatures (20–43 °C) under the experimental conditions. At 48 °C, however, incubation for 15 min rendered the enzyme virtually inactive. Another issue is with regard to the effects of divalent metal cations on the activity of the zebrafish ST. As an aquatic animal, zebrafish in the natural environment may be more vulnerable to the adverse effect of polluting heavy metal ions. Our previous studies had revealed that divalent metal cations can exert dramatic inhibitory/stimulatory effects on various human cytosolic STs [18,19,41]. Enzymic assays using DHEA as the substrate were

Figure 6 Effects of divalent metal cations on the sulphonating activity of the zebrafish ST

Purified zebrafish ST was assayed for its dopamine-sulphonating activity in the presence of different divalent metal cations or NaCl (as a control for the counter ion, Cl^-) under standard conditions as described in the Experimental section. The concentration of the divalent metal cations tested was 5 mM, and the concentration of NaCl tested was 10 mM.

carried out in the absence or presence of various divalent metal cations at a concentration of 5 mM. As a control for the counter ion, Cl^- , parallel assays in the presence 10 mM NaCl were also performed. Results obtained are shown in Figure 6. The degrees of inhibition or stimulation were calculated by comparing the activities determined in the presence of metal cations with the activities determined in the absence of metal cations. It was noted that the NaCl control exerted only a marginal inhibitory effect on the activity of the zebrafish ST. Among the ten divalent metal cations tested, Fe^{2+} and Cd^{2+} exhibited small, but significant, stimulatory effects, whereas Hg^{2+} and Cu^{2+} displayed considerably stronger inhibitory effects on the activity of the enzyme. More detailed studies will be required in order to fully elucidate the dose-dependence of the regulation of the activity of the zebrafish ST by these divalent metal cations and their modes of action.

In conclusion, the present study represents an endeavour aimed at identifying the cytosolic ST enzymes present in zebrafish. As mentioned earlier, the identification of the various cytosolic STs followed by their biochemical characterization is a prerequisite for using zebrafish as a model in a systematic investigation of some of the fundamental, and poorly understood, questions. More work is warranted in order to achieve this goal.

This work was supported in part by a Grant-in-Aid from the American Heart Association (Texas Affiliate), and a UTHCT President's Council Research Membership Seed Grant.

REFERENCES

- Mulder, G. J. and Jakoby, W. B. (1990) Sulfation. In *Conjugation Reactions in Drug Metabolism* (Mulder, G. J., ed.), pp. 107–161. Taylor and Francis Ltd., London.
- Falany, C. and Roth, J. A. (1993) Properties of human cytosolic sulfotransferases involved in drug metabolism. In *Human Drug Metabolism: From Molecular Biology to Man* (Jeffery, E. H., ed.), pp. 101–115. CRC Press Inc., Boca Raton, FL.
- Weinshilboum, R. and Otterness, D. (1994) Sulfotransferase enzymes. In *Conjugation–Deconjugation Reactions in Drug Metabolism and Toxicity* (Kaufmann, F. C., ed.), pp. 45–78. Springer-Verlag, Berlin.
- Coughtrie, M. W. H., Sharp, S., Maxwell, K. and Innes, N. P. (1998) Biology and function of the reversible sulfation pathway catalysed by human sulfotransferases and sulfatases. *Chem. Biol. Interact.* **109**, 3–27.
- Duffel, M. W. (1997) Sulfotransferases. In *Comprehensive Toxicology* (Guengerich, F. P., ed.), pp. 365–383. Elsevier Science Ltd., Oxford.
- Labrie, F., Belanger, A., Simard, J., Luu-The, V. and Labrie, C. (1995) DHEA and peripheral androgen and estrogen formation: intracrinology. *Ann. N.Y. Acad. Sci.* **774**, 16–28.
- Kroboth, P. D., Salek, F. S., Pittenger, A. L., Fabian, T. J. and Frye, R. F. (1999) DHEA and DHEA-S: a review. *J. Clin. Pharmacol.* **39**, 327–348.
- Corpechot, C., Robel, P., Axelson, M., Sjoval, J. and Baulieu, E. E. (1981) Characterization and measurement of dehydroepiandrosterone sulfate in rat brain. *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4704–4707.
- Mathur, C., Prasad, V. V., Raju, V. S., Welch, M. and Lieberman, S. (1993) Steroids and their conjugates in the mammalian brain. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 85–88.
- Baulieu, E. E. (1996) Dehydroepiandrosterone (DHEA): a fountain of youth? *J. Clin. Endocrinol. Metab.* **81**, 3147–3151.
- Baulieu, E. E. and Robel, P. (1998) Dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) as neuroactive neurosteroids. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 4089–4091.
- Schwartz, A. G. and Perantoni, A. (1975) Protective effect of dehydroepiandrosterone against aflatoxin B₁- and 7,12-dimethylbenz(a)anthracene-induced cytotoxicity and transformation in cultured cells. *Cancer Res.* **35**, 2482–2487.
- Barrett-Connor, E., Khaw, K. T. and Yen, S. S. (1986) A prospective study of dehydroepiandrosterone sulfate, mortality, and cardiovascular disease. *N. Engl. J. Med.* **315**, 1519–1524.
- Gordon, G. B., Bush, D. E. and Weisman, H. F. (1988) Reduction of atherosclerosis by administration of dehydroepiandrosterone. A study in the hypercholesterolemic New Zealand White rabbit with aortic intimal injury. *J. Clin. Invest.* **82**, 712–720.
- Mohan, P. F. (1989) Dehydroepiandrosterone and Alzheimer's disease. *Lancet* **II**, 1048–1049.
- Yamazoe, Y., Nagata, K., Ozawa, S. and Kato, R. (1994) Structural similarity and diversity of sulfotransferases. *Chem. Biol. Interact.* **92**, 107–117.
- Weinshilboum, R. M., Otterness, D. M., Aksoy, I. A., Wood, T. C., Her, C. T. and Raftogianis, R. B. (1997) Sulfation and sulfotransferases 1: sulfotransferase molecular biology: cDNAs and genes. *FASEB J.* **11**, 3–14.
- Sakakibara, Y., Suiko, M. and Liu, M.-C. (1994) De novo sulfation of L-tyrosine in HepG2 human hepatoma cells and its possible functional implication. *Eur. J. Biochem.* **226**, 293–301.
- Suiko, M., Sakakibara, Y., Nakajima, H., Sakaida, H. and Liu, M.-C. (1996) Enzymic sulphation of dopa and tyrosine isomers by HepG2 human hepatoma cells: stereoselectivity and stimulation by Mn^{2+} . *Biochem. J.* **314**, 151–158.
- Runge-Morris, M. and Wilusz, J. (1991) Age and gender-related gene expression of hydroxysteroid sulfotransferase- α in rat liver. *Biochem. Biophys. Res. Commun.* **175**, 1051–1056.
- Nagata, K., Ozawa, S., Miyata, M., Shimada, M., Gong, D. W., Yamazoe, Y. and Kato, R. (1993) Isolation and expression of a cDNA encoding a male-specific rat sulfotransferase that catalyzes activation of N-hydroxy-2-acetylaminofluorene. *J. Biol. Chem.* **268**, 24720–24725.
- Briggs, J. P. (2002) The zebrafish: a new model organism for integrative physiology. *Am. J. Physiol.* **282**, R3–R9.
- Ward, A. C. and Lieschke, G. J. (2002) The zebrafish as a model system for human disease. *Front. Biosci.* **7**, d827–d833.
- Yanagisawa, K., Sakakibara, Y., Suiko, M., Takami, Y., Nakayama, T., Nakajima, H., Takayanagi, K., Natori, Y. and Liu, M.-C. (1998) cDNA cloning, expression, and characterization of the human bifunctional ATP sulfurylase/adenosine 5'-phosphosulfate kinase enzyme. *Biosci. Biotechnol. Biochem.* **62**, 1037–1040.
- Liu, M.-C. and Lipmann, F. (1984) Decrease of tyrosine-O-sulfate-containing proteins found in rat fibroblasts infected with Rous sarcoma virus or Fujinami sarcoma virus. *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3695–3698.
- Fernando, P. H. P., Karakawa, A., Sakakibara, Y., Ibuki, H., Nakajima, H., Liu, M.-C. and Suiko, M. (1993) Preparation of 3'-phosphoadenosine 5'-phospho[^{32}S]sulfate using ATP sulfurylase and APS kinase from *Bacillus stearothermophilus*: enzymatic synthesis and purification. *Biosci. Biotechnol. Biochem.* **5**, 1974–1975.
- Lin, E.-S. and Yang, Y.-S. (2000) Nucleotide binding and sulfation catalyzed by phenol sulfotransferase. *Biochem. Biophys. Res. Commun.* **271**, 818–822.
- Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685.
- Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.
- Lipmann, F. (1958) Biological sulfate activation and transfer. *Science* **128**, 575–580.
- Chang, H. J., Zhou, M. and Lin, S. X. (2001) Human dehydroepiandrosterone sulfotransferase: purification and characterization of a recombinant protein. *J. Steroid Biochem. Mol. Biol.* **77**, 159–165.
- Geese, W. J. and Raftogianis, R. B. (2001) Biochemical characterization and tissue distribution of human SULT2B1. *Biochem. Biophys. Res. Commun.* **288**, 280–289.
- Meloche, C. A. and Falany, C. N. (2001) Expression and characterization of the human 3 β -hydroxysteroid sulfotransferases (SULT2B1a and SULT2B1b). *J. Steroid Biochem. Mol. Biol.* **77**, 261–269.

-
- 34 Baulieu, E. E. (1997) Neurosteroids: of the nervous system, by the nervous system, for the nervous system. *Recent Prog. Horm. Res.* **52**, 1–32
- 35 Plassart-Schiess, E. and Baulieu, E. E. (2001) Neurosteroids: recent findings. *Brain Res. Rev.* **37**, 133–140
- 36 Shimada, M., Yoshinari, K., Tanabe, E., Shimakawa, E., Kobashi, M., Nagata, K. and Yamazoe, Y. (2001) Identification of ST2A1 as a rat brain neurosteroid sulfotransferase mRNA. *Brain Res.* **920**, 222–225
- 37 Jain, M. R., Khan, F. A., Krishna, N. S. and Subhedar, N. (1994) Intracranial metyrapone stimulates CRF-ACTH axis in the teleost, *Clarias batrachus*: possible role of neurosteroids. *Neuroreport* **5**, 2093–2096
- 38 Mathieu, M., Mensah-Nyagan, A. G., Vallarino, M., Do-Rego, J. L., Beaujean, D., Vaudry, D., Luu-The, V., Pelletier, G. and Vaudry, H. (2001) Immunohistochemical localization of 3- β -hydroxysteroid dehydrogenase and 5- α -reductase in the brain of the African lungfish *Protopterus annectens*. *J. Comp. Neurol.* **438**, 123–135
- 39 Parker, Jr, C. R., Falany, C. N., Stockard, C. R., Stankovic, A. K. and Grizzle, W. E. (1994) Immunohistochemical localization of dehydroepiandrosterone sulfotransferase in human fetal tissues. *J. Clin. Endocrinol. Metab.* **78**, 234–236
- 40 Westerfield, M. (2000) *The Zebrafish Book*, University of Oregon Press, Eugene, OR
- 41 Xu, F., Suiko, M., Sakakibara, Y., Pai, T. G. and Liu, M.-C. (2002) Regulatory effects of divalent metal cations on human cytosolic sulfotransferases. *J. Biochem. (Tokyo)* **132**, 457–462

Received 11 July 2003; accepted 29 July 2003

Published as BJ Immediate Publication 29 July 2003, DOI 10.1042/BJ20031050