Characterization of iodothyronine sulfotransferase activity in the cytosol of *Rana catesbeiana* tadpole tissues

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Abstract

We have investigated the sulfation of thyroid hormones (THs) in the cytosol from *Rana catesbeiana* tadpole tissues. Sulfation of 3,3',5-triiodothyronine (T_3) by the liver cytosol, which was dependent on protein amount, incubation time, and temperature, suggested the presence of TH sulfotransferases (SULTs) in the liver. The apparent Michaelis–Menten constant (K_m) of the liver cytosol was 0.22 μM for T_3, and the apparent maximum velocity (V_{max}) of the liver cytosol was 7.65 pmol/min/mg protein for T_3. Iodothyronine sulfating activity in the liver cytosol was increased in tadpoles at premetamorphic (stages IX–X) and metamorphic climax (stage XX) stages, and in adult frogs. The substrate preference of iodothyronine sulfation for the liver cytosol from tadpoles (stage X) was: 3,3',5'-triiodothyronine > T_3 > 3,3',5,5'-tetraiodothyroacetic acid > 3,3',5-triiodothyroacetic acid, T_4, 3-iodothyronine > 3,5-diiodothyronine. Several halogenated phenols were potent inhibitors (IC_{50} = 0.15–0.21 μM). The substrate preference for T_3 was gradually lost by the onset of metamorphic climax stages. These enzymatic characteristics of iodothyronine sulfation in the liver cytosol from tadpoles resembled those of mammalian phenol SULTs, except that the tadpole cytosol had a higher affinity (one or two orders of magnitude) for T_3 than mammalian SULTs. These results suggested that an enzyme homologous to mammalian phenol SULT (SULT1) may be involved in TH metabolism in tadpoles.

Keywords: iodothyronine; sulfotransferase; metamorphosis; liver; *Rana catesbeiana*

1. **Introduction**

Thyroid hormones (THs, iodothyronines) are iodinated amino acid-derived hormones, most of which are secreted as thyroxine (L-3,3',5,5'-tetraiodothyronine, T_4), from the thyroid gland into the bloodstream. In peripheral tissues, T_4 is metabolized to 3,3',5-triiodothyronine (T_3), an active form of TH, by outer ring deiodinase (5'D) or 3,3',5'-triiodothyronine (rT_3), an inactive form of TH, by inner ring deiodinase (5D). THs are also metabolized by nondeiodination pathways, such as sulfation, glucuronidation, ether link cleavage, deamination, and decarboxylation (Wu et al., 2005). Of these pathways, iodothyronine sulfation in mammalian species has been a key research focus (Visser, 1996; Darras et al., 1999). However, little is known about the extent to which sulfation contributes to TH metabolism in amphibians, especially during metamorphosis, which is obligatorily regulated by THs. The study of the sulfation by *Xenopus laevis* oocyte cytosol suggested the presence of sufotransferases (SULTs) with a substrate preference for rT_3 (Friesema et al., 1998). This is different from the characteristics of the mammalian SULTs (Young et al., 1988; Gong et al., 1992; Kaptein et al., 1997; Visser et al., 1998).

Sulfation of iodothyronines is catalyzed by cytosolic sulfotransferases (SULTs), a supergene family of important phase II conjugation enzymes. In mammals, SULTs comprise five distinct gene families (SULT1, SULT2, SULT3, SULT4 and SULT5) (Gamage et al., 2006). Sulfotransferases consist of two subunits (molecular mass is 31–35 kDa for each subunit) (Stanley et al., 2005). Cytosolic SULTs are usually present as homodimers in various tissues, such as the liver, kidney, intestine, skin, and brain depending on the families or subfamilies (Strott, 2002; Gamage et al., 2006). Using 3'-phosphoadenosine-5'-phosphosulfate (PAPS) as a sulfate donor, SULTs detoxify various xenobiotics and modulate the activity of endogenous hormones, bile acids, and neurotransmitters, e.g., phenols, iodothyronines, estrogens and catecholamines in the SULT1 family, neutral steroids and sterols in the SULT2 family, and heterocyclic amines in the SULT3 family (Blanchard et al., 2004; Gamage et al., 2006; Wang and James, 2006; Testa and Krämer, 2008). The human SULT1A subfamily (SULT1A1, SULT1A2, and SULT1A3 isoforms), SULT1B subfamily

(SULT1B1 isoform), SULT1C subfamily (SULT1C2 isoform), SULT1E subfamily (SULT1E1 isoform), and SULT2A subfamily (SULT2A1 isoform) all catalyze iodothyronine sulfation (Wu et al., 2005) and are tissue specific. Of these SULT isoforms, SULT1A1 is the most abundant in the liver, when compared with the other SULT isoforms, and has a relatively lower Michaelis–Menten constant (K_m) for T_3 than the other SULT isoforms (Young et al., 1988; Kester et al., 1999), whereas SULT1B1, the major iodothyronine SULT found in the small intestine and colon, has a K_m for T_3 lower than SULT1A1 and SULT1A3 (Wang et al. 1998; Fujita et al., 1999).

The biochemical properties and physiological functions of iodothyronine SULTs in the liver of lower vertebrates are distinct from those in the liver of higher vertebrates. Of the iodothyronines, major mammalian liver iodothyronine SULTs have a substrate preference for 3,3'-diiodothyronine (3,3'-T_2; rank order: 3,3'-T_2 > rT_3 > T_3 > T_4 in humans, and 3,3'-T_2 > T_3 > rT_3 > T_4 in rats) (Gong et al., 1992; Visser, 1994; Visser et al., 1998; Kester et al., 1999; Wu et al., 2005). Interestingly, in rat, sulfation of these iodothyronines, except for rT_3, accelerates (by 40- to-200-fold) deiodination, and initiates the irreversible degradation, of THs (Visser, 1996). However, when deiodinase activity is low, i.e., during fetal development and non-thyroidal illness (Chopra, 2004), iodothyronine sulfation is reversible, inactivating THs and allowing T_4 and T_3 sulfates (T_4S and T_3S) accumulate as a TH reservoir in plasma (Visser, 1994; Visser et al., 1996; Darras et al., 1999; Wu et al., 2005). In contrast, fish liver iodothyronine SULTs have a substrate preference for rT_3 (rank order: rT_3 >> T_3 =T_4 = 3,5-T_2) (Finnson and Eales, 1998) and iodothyronine sulfation in fish inhibits TH deiodination (Finnson et al., 1999).

Information about the role of iodothyronine SULTs in amphibians, particularly during development, however, is lacking. In amphibian metamorphosis, the activation and inactivation of THs by deiodinases plays a central role in the sensitivity of peripheral tissues to, and timing of, TH actions (Brown, 2005). Findings from studies of THs and their metabolites, deiodinases (Morvan Dubois et al., 2006), $^{125}I^-$ uptake

96 (Tindall et al., 2007), and TH receptor mRNA (Banker et al., 1991) in amphibian embryos or larvae raise
97 the possibility that TH signalling occurs before the development of the thyroid gland.
98 In this study, we aim to elucidate if the enzymatic properties of iodothyronine SULT(s) exist in cytosol
99 from *Rana catesbeiana* tadpoles and the liver cytosol from *R. catesbeiana* adult bullfrogs.
100

2. Materials and Methods

2.1. Reagents

[5'-^{125}I]T_4 (35.9 TBq/mmol) was purchased from PerkinElmer (Waltham, MA, USA). Unlabeled T_3 (>97% purity), T_4 (>98% purity), rT_3 (>97% purity), 3,3',5,5'-tetraiodothyroacetic acid (Tetrac, >98% purity), 3,3',5-triiodothyroacetic acid (Triac, ~95% purity), pentachlorophenol (99% purity), and PAPS (83% purity) were obtained from Sigma-Aldrich (St. Louis, MO, USA). 3-Iodothyronine (T_1, >97% purity) was purchased from Toronto Research Chemicals (Toronto, Canada). Ioxynil (3,5-diiodo-4-hydroxybenzonitrile, analytical standard, 99% purity) was obtained from Riedel-de Haën Fine Chemicals (Seelze, Germany). Diethylstilbestrol (>98% purity), 3,5- T_2 (>97% purity), 3,3',5,5'-tetrabromobisphenol A (>98% purity), and 3,3',5,5'-tetrachlorobisphenol A (>98% purity) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Pentabromophenol (98% purity) was from Alfa Aesar (Heysham, Lancashire, UK). 2,4,6-Triiodophenol (98% purity), 2,4,6-tribromophenol (98% purity), 2,4,6-trichlorophenol (97% purity), 2,6-dichlorophenol (95% purity), 2,6-dibromophenol (98% purity), 2,6-dichloro-4-nitrophenol (97% purity), *p*-nitrophenol (99% purity), dopamine (95% purity), dihydroxyepiandrosterone (DHEA, 97% purity), 4-nonylphenol (99% purity) and 17β-estradiol (E_2, >97% purity) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Sephadex LH-20 resin was obtained from GE Healthcare (Uppsala, Sweden). All other chemicals used in this study were of the highest grade available and were purchased from Wako Pure Chemical Industries or Nacalai Tesque (Kyoto, Japan).

Immediately before use, [^{125}I]T_4 was purified using a Sephadex LH-20 column. [^{125}I]T_3 and [^{125}I]T_4 were purchased or prepared in our laboratory by radioiodination using the chloramine-T method (Greenwood et al., 1963), with slight modifications. [^{125}I]T_3 and [^{125}I]T_4 were purified by reverse phase high-performance liquid chromatography with a isocratic mobile phase (methanol:distilled water:acetic acid, 54.5:44.5:1) at 1 mL/min at 40°C , with retention times of 19.1 min for [^{125}I]T_3 and of 25.1 min for

125 [^{125}I]T_4. The specific activities were 1.01–1.75 TBq/mmol for [^{125}I]T_3 and 1.52–1.55 TBq/mmol for [^{125}I]T_4.
126 The purity of [^{125}I]T_3 and [^{125}I]T_4 was confirmed by thin layer chromatography on silica gel (PE Sil G/UV,
127 Whatman; Maidstone, UK) (Chanoine et al., 1992) using chloroform:methanol:25% ammonia (55:40:5) as
128 a solvent, followed by autoradiography. The R_f values were 0.87 for iodine, 0.41 for T_4, and 0.24 for T_3.
129 All potential inhibitors and substrates of iodothyronine SULTs were dissolved in dimethyl sulfoxide or
130 0.1 M sodium phosphate, pH 7.2, to concentrations ranging from 2 to 10 mM. These chemicals were
131 diluted with phosphate buffer (pH 7.2), to give less than 0.1% (v/v) solvent.
132
133 *2.2. Animals*
134 *Rana catesbeiana* tadpoles at stages IX–XXIV (Taylor and Kollros, 1946), froglets and adults frogs
135 (male and female) were collected from ponds in Shizuoka Prefecture, Japan, or in Saitama Prefecture,
136 Japan, from March 2007 to July 2008. The tadpoles were maintained in aerated, dechlorinated tap water at
137 20–25°C, unless otherwise noted, and fed boiled spinach three times a week.
138
139 *2.3. Preparation of cytosol*
140 Tadples and froglets (8–18 g body weight) were anesthetized by immersion in 0.2% (w/v) ethyl
141 3-aminobenzoate methanesulfonic acid (Sigma-Aldrich) whereas adult frogs (170–200 g body weight) were
142 pithed, in accordance with the code of ethics on the Animal Welfare Committee of Shizuoka University.
143 Liver was removed and immediately used for the preparation of cytosol.
144 Froglets and adult frog tissues were perfused with ice-cold frog Ringer (111 mM NaCl, 3.4 mM KCl,
145 2 mM $CaCl_2$, 2.3 mM $NaHCO_3$) containing 0.2 mg/mL heparin. After determining the tissue mass, the
146 tissue was minced with scissors. Tadpole tissues were minced directly with scissors without perfusion and
147 then extensively rinsed with ice-cold frog Ringer to remove plasma and blood cells. Minced tissues were
148 homogenized in 4.5 vol. of 0.25 M sucrose, 10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid

(EDTA), 1 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM benzamidine hydrochloride, and 1 mM phenylmethanesulfonyl fluoride, pH 7.5, using a glass Teflon homogenizer. The crude homogenate was centrifuged at 1,200 × *g* for 15 min at 4˚C to remove the nuclear pellet. The post-nuclear supernatant was further centrifuged at 12,000 × *g* for 20 min at 4˚C to separate the crude mitochondrial/lysosomal pellet from the supernatant. The resulting post-mitochondrial/lysosomal supernatant was centrifuged at 105,000 × *g* for 2 h at 4˚C to obtain the clear supernatant (cytosol), which was stored in 10% glycerol at –80˚C until required. The protein concentration was determined by the Bradford (1976) method, using γ-globulin as the standard.

2.4. Assay of iodothyronine SULT activity

Iodothyronine sulfating activity was measured as described (Young et al.,1988; Kaptein et al., 1997). Briefly, 1 μM T_3 containing 100,000 dpm [^{125}I]T_3 or 1 μM T_4 containing 100,000 dpm [^{125}I]T_4 was incubated with 0.3 mg/mL cytosolic proteins in the presence (complete) or absence (blank) of 50 μM PAPS in 0.2 mL of 0.1 M sodium phosphate, pH 7.2, and 2 mM EDTA for 30 min at 16˚C. The reaction was started by adding the cytosol and was stopped by adding 0.8 mL 0.1 N HCl to the reaction mixture. The formation of iodothyronine sulfate was analyzed using Sephadex LH-20 chromatography on a mini-column (bed volume, 0.75 mL), pre-equilibrated with 0.1 N HCl, as described (Rooda et al., 1987). Free iodine, sulfated iodothyronines, and nonsulfated iodothyronines were eluted successively using six 1 mL aliquots of 0.1 N HCl, ten 1 mL aliquots of distilled water and six 1 mL aliquots of 0.2 M ammonia/ethanol (1/1, vol/vol) (Rutgers et al., 1987). One-mL fractions were collected and their radioactivity was measured in a gamma counter (Auto Well Gamma System ARC-380CL, Aloka, Japan). Iodothyronine sulfation was estimated by subtracting the radioactivity of the blank samples (without PAPS) from that of the complete samples (with PAPS).

2.5. Statistical analysis

The data are presented as the mean ± standard error of mean (n = 3), unless otherwise noted. All experiments were repeated independently three times. Where appropriate, differences between two groups were evaluated statistically by a two-way analysis of variance followed by Fisher's least-significant-different method for multiple comparisons. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Assay conditions of iodothyronine sulfation by tadpole liver cytosol

Our findings suggest that the liver cytosol from *R. catesbeiana* tadpoles (stage X) contains TH sulfating activity. In terms of the amount of protein in the liver cytosol, T_3 sulfating activity increased steadily as the protein amount (up to 0.24 mg) increased (Fig. 1A). In terms of incubation time, T_3 sulfation increased linearly for at least 90 min (Fig. 1B). In terms of temperature, T_3 sulfation increased gradually as temperature increased before reaching a plateau at about 22˚C (Fig. 1C).

3.2. Enzyme kinetics

Saturation kinetics was demonstrated for T_3 sulfation by the liver cytosol from *R. catesbeiana* tadpoles (stage X) at varying concentrations (0–16 μM) of T_3 and a fixed concentration (50 μM) of PAPS (Fig. 2A). The double-reciprocal plot of sulfation rates *vs*. T_3 concentration was linear (Fig. 2A, *inset*), with an apparent K_m of 0.22 μM and an apparent V_{max} of 7.65 pmol/min/mg protein. Saturation kinetics was also demonstrated when T_3 sulfation was measured at a fixed concentration (20 μM) of T_3 and various concentrations (0–10 μM) of PAPS (Fig. 2B). From the double-reciprocal plot (Fig. 2B, *inset*), the apparent K_m for PAPS was 2.41 μM and the apparent V_{max} was 99.2 pmol/min/mg protein. When T_4 sulfation by the liver cytosol from *R. catesbeiana* tadpoles was investigated using the same assay procedure, the rate of T_4 sulfation was lower than that of T_3 sulfation. The substrate specificity of the tadpole liver cytosol sulphating activity as expressed by V_{max}/K_m value was 11-fold greater for T_3 (34.8) than that for T_4 (3.10) (Table 1).

3.3. Temporal and spatial expression patterns of TH sulfating activity in tadpole cytosol

The pattern of liver TH sulfating activity during development was of particular interest because of three distinct stages of activity (Fig. 3A). T_4 sulfating activity in liver cytosol was highest in adult frogs,

followed by tadpoles at the beginning of the metamorphic climax stage (stage XX) and at the premetamorphic stage (stage X). Liver cytosol from tadpoles at other stages had relatively low T_4 sulfating activity. T_3 sulfating activity in liver cytosol was highest in tadpoles at the premetamorphic stages (stages IX and X), followed by adult frogs then tadpoles at the prometapmorphic stages (Stages XV, XVIII and XX). In adult frogs, both T_4 and T_3 sulfating activities were slightly higher in males than in female. The T_3:T_4 sulfating activity ratio was high (about 4.5) at stage IX, decreased during the premetamorphic (stage X) and prometamorphic (stages XV and XVIII) stages to a minimum (about 1) before the metamorphic climax stages (stage XX) (Fig. 3A, *inset*). This ratio was maintained in the liver cytosol from froglets, and adult frogs. In terms of tissue-specific TH sulfating activities in cytosol from tadpoles (stage X), T_4 and T_3 sulfating activities were highest in the liver, followed by the kidney, intestine, and brain (Fig. 3B).

3.4. Substrate-specificity of T_3 sulfating activity

Of the iodothyronines and their analogs investigated, rT_3 was the most potent competitor of T_3 sulfation in liver cytosol from tadpoles (stage X) (50% inhibitory concentration [IC_{50}] = 83±3 nM; Fig. 4A). The rank order of potency was rT_3 > T_3 (IC_{50} = 130±0 nM) > Tetrac (IC_{50} = 830±30 nM) > Triac (IC_{50} = 1930±60 nM), T_4, T_1> 3,5-T_2. When a similar experiment was done using the liver cytosol from adult frogs, inhibitory potency of T_3 on [^{125}I]T_3 sulfation was as low as that of T_4 (Fig. 4A). Of the mammalian SULT substrates investigated, DHEA, diethylstilbestrol, *p*-nitrophenol, dopamine, and E_2 (all at 1μM) inhibited T_3 sulfation by the liver cytosol by 55%, 42%, 20%, 13%, and 10%, respectively (Fig. 4B). The IC_{50} for DHEA was 860±70 nM.

3.5. Inhibitions of iodothyronine sulfating activity

Of the halogenated phenol and phenolic compounds tested, pentachlorophenol, pentabromophenol and triiodophenol were potent inhibitors of T_3 sulfation by the liver cytosol from tadpoles. Pentachlorophenol

was the most potent competitor (IC_{50} = 150±10 nM; Fig. 5A). The rank order of inhibition potency was pentachlorophenol > pentabromophenol (IC_{50} = 160±30 nM) > triiodophenol (IC_{50} = 210±20 nM) > 2,6-dichlorophenol (IC_{50} = 360±20 nM) > trichlorophenol (IC_{50} = 370±30 nM) > 2,6-dichloro-4-nitrophenol (IC_{50} = 490±10 nM)> 2,6-dibromophenol (IC_{50} = 550±30 nM) > tribromophenol (IC_{50} = 800±50 nM) > ioxynil (IC_{50} = 970±30 nM). Tetrabromobisphenol A and tetrachlorobisohenol A (halogenated phenolic compounds consisting of two benzene rings) were also potent inhibitors (inhibition at 1 μM = 52 and 45%, respectively; IC_{50} for tetrabromobisphenol A = 970±30 nM) (Fig. 5B). However, nonylphenol and bisphenol A (non-halogenated phenol and phenolic compounds) had little effect on T_3 sulfation (inhibiton at 1μM = 4% and 9%, respectively).

4. Discussion

In this study, we have characterized the biochemical properties of iodothyronine sulfating activity in the liver cytosol from *R. catesbeiana* tadpoles. Our findings suggest that the affinity of the enzyme in the tadpole liver cytosol for T_3 was higher than those of the enzymes in the liver cytosol from other species. As 2,6-dichloro-4-nitrophenol and other halogenated phenols, dopamine, E_2, and DHEA inhibited significantly [^{125}I]T_3 sulfation by the liver cytosol, we believe that a phenol SULT(s) belonging to the SULT1 family may be responsible for the majority of this activity.

Our findings indicate that the tadpole iodothyronine SULT(s) might have adapted to metabolize T_3 more quickly at low concentrations than other mammalian SULTs. Compared with mammalian SULTs, liver cytosol from tadpoles had (i) a higher affinity for T_3; (ii) an apparent K_m for T_3 that was two orders of magnitude lower (48–81 μM *vs* 0.22 μM; Young et al., 1988; Kaptein et al., 1997; Visser et al., 1998; Kester et al., 1999); (iii) a V_{max}/K_m ratio for T_3 one or two orders of magnitude higher (0.4–4.6 *vs* 34.8; Kaptein et al., 1997; Visser et al. 1998; Kester et al., 1999); and (iv) a V_{max}/K_m ratio for T_3 was one order of magnitude lower than for 3,3'-T_2 (300–1100 *vs* 34.8; Kaptein et al. 1997; Visser et al. 1998; Kester et al., 1999; Kester et al., 2003). In contrast, the V_{max}/K_m ratio for PAPS of the liver cytosol from tadpoles was similar to that of mammalian SULTs (Young et al., 1988; Kaptein et al., 1997; Kester et al. 1999).

The liver cytosol from tadpoles is remarkable for its relatively high TH sulfating activity at stage XX, after which TH-dependent remodeling occurs in the liver. However, developmental changes in iodothyronine sulfating activity are likely to be species specific. TH sulfating activity was high in the liver cytosol from *R. catesbeiana* tadpoles at premetamorphic (stages IX–X) and metamorphic climax stages (stage XX) and from adults. In the liver from axolotls, 3,3'-T_2 sulfating activity was highest during premetamorphosis and decreased gradually during metamorphosis (Reyns et al., 2000). In contrast, the expression of the zebrafish iodothyronine SULT (SULT1AST5) was observed at the beginning of the hatching period during embryogenesis, and gradually increased throughout the larval stage into maturity

261 (Yasuda et al., 2005).

262 Of the iodothyronines and their analogs, rT_3 and T_3 were the preferred substrates for sulfation by the
263 liver cytosol from tadpoles. This substrate preference resembles that of the zebrafish iodothyronine SULT
264 (SULT1AST5), which belongs to the SULT1 family (Yasuda et al., 2005). Except for the its preference for
265 T_3, the liver cytosol from tadpoles had a rank order of the potency that was similar to those for mammalian
266 liver SULT1 family enzymes (Visser et al., 1998; Kester et al., 1999). In contrast, the substrate preference
267 of enzymes obtained from chicken liver (Reyns et al., 2000), axolotl and *Xenopus* oocytes (Friesema et al.,
268 1998; Reyns et al., 2000), and rainbow trout liver (Finnson & Eales, 1998) was extremely higher for rT_3
269 than for T_3. Given that the liver cytosol from tadpoles has the IC_{50} value for rT_3 that was merely 1.6 times
270 less than that for T_3, the tadpole liver may catalyze T_3 sulfation as well as rT_3 sulfation before metamorphic
271 climax stages. 3,3'-T_2 is also one of the preferred substrates of mammalian iodothyronine SULTs. Although
272 we did not investigate 3,3'-T_2 sulfation by the liver cytosol from tadpoles, 3,3'-T_2S has been detected with
273 rT_3S, but not with T_3S, in the plasma of metamorphosing tadpoles (stages X and XX) (Wu et al., 1998).
274 This suggests that 3,3'-T_2 may also be a preferred substrate of tadpole SULTs.

275 Results from this study suggest that the liver cytosol from tadpoles, like that from mammals, may
276 contain more than one SULT isoform that may have more than one substrate. In mammals and zebrafish,
277 several SULTs belonging to the SULT1and SULT2 families have iodothyronine sulfating activity (Visser,
278 1996; Wu et al., 2005; Gamage et al., 2006; Sugawara et al., 2003a; 2003b; Yasuda et al., 2005). In humans,
279 there are several SULT isoforms that sulfate more than one substrate and have overlapping substrate
280 specificity, e.g., SULT1A1 sulfates simple phenols, iodothyronines, and estrogens (Stanley et al., 2005;
281 Gamage et al., 2006). We found that the T_3:T_4 sulfating activity ratio decreased during development from
282 premetamorphosis to prometamorphosis and that competition between T_3 and [^{125}I]T_3 sulfation by the
283 tadpole liver cytosol was one order of magnitude higher than that by the adult liver cytosol. These results
284 suggest that a SULT isoform with high T_3 preference may exist in the liver cytosol of tadpole during

premetamorphosis, and may be displaced by another SULT isoform with a low T_3 preference during metamorphosis climax. In addition, we demonstrated that the liver cytosol from tadpoles sulfated more than one substrate, including *p*-nitrophenol, dopamine, E_2, and DHEA, as well as iodothyronines.

Iodothyronine SULT(s) in *R. catesbiana* tadpoles may be a molecular target for chemicals known to disrupt the amphibian thyroid system. Amphibian tadpoles have a thin, permeable skin and inhabit aquatic environments. As a result, they are susceptible to contaminants in the wastewater discharges from agricultural fields, and industrial and household areas. Our previous studies indicated that pentachlorophenol, triiodophenol, ioxynil, 3,3',5,5'-tetrabromobisphenol A, and 3,3',5,5'-tetrachloroobisphenol A affect the amphibian thyroid system by interfering with either TH binding to plasma proteins or TH nuclear receptors *in vivo* and *in vivo* (Ishihara et al., 2003; Kudo and Yamauchi, 2005; Kudo et al., 2006). In this study, [^{125}I]T_3 sulfation by the tadpole liver cytosol was strongly inhibited by halogenated phenols and phenolic compounds. 2,6-Dichloro-4-nitrophenol, an inhibitor of mammalian SULT1A1, inhibited T_3 sulfation by the liver cytosol from tadpoles; inhibition was one order of magnitude lower than that by the liver cytosol from mammals (Young et al., 1988; Gong et al., 1992) or recombinant SULT1A1 (Fujita et al., 1999). Simple phenols (pentachlorophenol, pentabromophenol, and 2,4,6-triiodophenol) were the most potent inhibitors, whereas phenolic compounds (3,3',5,5'-tetrabromobisphenol A and 3,3',5,5'-tetrachlorobisphenol A) were moderate inhibitors of T_3 sulfation by liver cytosol from tadpoles. In addition, these compounds inhibit 3,3'-T_2 or T_3 sulfation by mammalian and rainbow trout liver (Visser et al., 1998; Gong et al., 1992; Schuur et al., 1998; Finnson and Eales, 1998).

Given our findings, we propose two possible pathways of iodothyronine metabolism in the tadpole liver (Fig. 6). As iodothyronines in the tadpole plasma increase to detectable concentrations during premetamorphosis (stage XIII), peak during metamorphosis climax (stages XX–XXII), then gradually decrease (White and Nicoll, 1981), T_4 and T_3 would need to be metabolized quickly to rT_3S [Pathway (I)]

309 and to 3,3'-T_2S [Pathway (II)], respectively, by both 5D and SULT(s) before metamorphic climax stages.
310 In both pathways, two routes are possible. To know which route is predominant, we would need to
311 determine whether the *R. catesbeiana* 5D, like the mammalian 5D (Visser, 1996), prefers T_4S and T_3S,
312 rather than T_4 and T_3 as substrates. The tadpole liver has high activity of 5D until stage XIX, but no activity
313 of 5'D, which is expressed in the intestine, tail, leg, eye, and skin in a tissue-specific manner during
314 metamorphosis (Becker et al., 1997). *R. catesbeiana* 5D has an affinity for T_3 one order of magnitude
315 higher than for T_4 (Galton and Hiebert, 1987). These pathways would protect the tadpole liver against
316 excess THs before the metamorphic climax stages, and would need to be fine-tuned for the onset of
317 T_3-dependent remodeling at a defined metamorphic climax stage. The concentrations of T_4S and T_3S are
318 negligible in tadpole plasma (Wu et al., 1998), unlike those in mammalian fetal plasma (Visser, 1994;
319 1996; Wu et al., 1998; Darras et al., 1999; Wu et al., 2005). Therefore, iodothyronine sulfation in the
320 tadpoles would not provide a TH reservoir comparable to those in mammalian fetuses, in the forms of T_4S
321 and T_3S from which active THs are recovered by sulfatases (Hazenberg et al., 1988).
322 In conclusion, we discovered T_3 sulfation activity with a low K_m in the liver cytosol from *R.*
323 *catesbeiana* tadpole that decreased during premetamorphic and prometamorphic stages. The enzymatic
324 characteristics of this activity, in terms of substrate preference and sensitivity to inhibitors, resemble those
325 of mammalian phenol SULTs belonging to the SULT1A family. Our findings suggest that the potential
326 SULT(s) in the liver of tadpoles may play a role in iodothyronine metabolism and cellular actions
327 modulated by THs at low concentrations before metamorphic climax (stage X).
328

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450

451

452 **Figure legends**

453 Figure 1. Iodothyronine sulfation by the liver cytosol from *Rana catesbeiana* tadpoles. (*A*)
454 Cytosol-dependent T_3 sulfation. The cytosol from the stage X tadpoles was incubated with T_3 (1 μM)
455 containing [^{125}I]T_3, and PAPS (50 μM) in 0.2 mL of the buffer, pH 7.2, for 30 min at 16˚C. (*B*) Time course
456 of T_3 sulfation. The cytosol was incubated with T_3 (1 μM) containing [^{125}I]T_3, and PAPS (50 μM) in 0.2 mL
457 of the buffer, pH 7.2, for up to 90 min at 16˚C. (*C*) Temperature-dependency of T_3 sulfation. The liver
458 cytosol was incubated with T_3 (1 μM) containing [^{125}I]T_3, and PAPS (50 μM) in 0.2 mL of the buffer, pH
459 7.2, for 30 min at 10˚C, 16˚C, 22˚C, and 28˚C. Values are the mean ± standard error of the mean. Each
460 experiment was repeated three times.
461
462 Figure 2. Kinetics of T_3 sulfation by the liver cytosol from *Rana catesbeiana* tadpoles. (*A*) T_3
463 concentration-dependent T_3 sulfation. The cytosol from the stage X tadpoles was incubated with T_3
464 (0–2 μM) containing [^{125}I]T_3 and PAPS (50 μM) for 30 min at 16˚C. (*B*) PAPS concentration-dependent T_3
465 sulfation. The cytosol was incubated with T_3 (20 μM) containing [^{125}I]T_3 and PAPS (0–10 μM) for 30 min
466 at 16˚C. Curve fitting was performed using the Michaelis–Menten equation, $v=V_{max}/(1+K_m/S)$. *Insets*
467 illustrate the double reciprocal plot. Values are the mean ± standard error of the mean. Experiments were
468 repeated three times.
469
470 Figure 3. Temporal and spatial patterns of TH sulfating activity in *Rana catesbeiana* cytosol. (*A*) TH
471 sulfation by liver cytosol during development. The cytosol from the metamorphosing tadpoles (stages
472 IX–XXIV), froglets, and adults were used for T_4 and T_3 sulfating activities (open and closed bars,
473 respectively). The *inset* depicts T_3:T_4 sulfating activity ratio. (*B*) TH sulfation by the cytosols from various

474 tissues of tadpoles (stage X). Each value is the mean ± SEM of triplicate determinations. Different letters
475 indicate significance between two time points (p <0.05; two-way analysis of variance followed by
476 Fisher's least-significant-difference method for multiple comparisons). P<0.05, between T_4 and T_3
477 sulfating activities in panels *A* and *B.* Experiments were repeated three times.
478
479 Figure 4. Substrate specificity of iodothyronine sulfating activity in the liver cytosol from *Rana*
480 *catesbeiana* tadpoles. (*A*) Competition of [^{125}I]T_3 sulfation by the liver cytosol with iodothyronines and
481 their analogs. Substrates investigated are 3,3',5'-triiodothyronine (rT_3, ●), T_3 (○),
482 3,3',5,5'-tetraiodothyroacetic acid (Tetrac, ▲), 3,3',5-triiodothyroacetic acid (Triac, <open triangle>),
483 thyroxine (T_4, ■), 3-iodothyronine (T_1, □); 3,5-diiodothyronine (3,5-T_2, ♦). For comparison, broken lines
484 indicate competition curves obtained using the liver cytosol from the adult frogs. (*B*) Competition of
485 [^{125}I]T_3 sulfation by the liver cytosol with candidate substrates. Substrates investigated are
486 dihydroxyepiandrosterone (DHEA, ●), diethylstilbestrol (DES, ○); *p*-nitrophenol (p-NiP,▲); dopamine
487 (<open triangle>); 17β-estradiol (E_2, ■). Values are the mean ± standard error of the mean. Experiments
488 were repeated three times.
489
490 Figure 5. Inhibition of [^{125}I]T_3 sulfating activity in the tadpole liver cytosol by halogenated phenol (*A*) and
491 phenolic compounds (*B*). Compounds investigated were pentachlorophenol (PCP, ●), pentabromophenol
492 (PBP, ○), triiodophenol (TIP, ▲), 2,6-dichlorophenol (DCP, <open triangle>), trichlorophenol (TCP, ■),
493 2,6-dichloro-4-nitrophenol (DCNP, □), 2,6-dibromophenol (DBP,♦), tribromophenol (TBP, ◊), ioxynil (▼)
494 and 4-nonylphenol (4-NoP, <upside-down open triangle>) in *panel A*; and tetrabromobisphenol A (Br_4BPA,
495 ○), tetrachlorobisphenol A (Cl_4BPA, <open triangle>) and bisphenol A (BPA, □) in *panel B*. Values are the
496 mean ± standard error of the mean. Experiments were repeated three times.
497

498 Figure 6. Possible pathways of iodothyronine metabolism in the tadpole liver. As outer ring deiodinase
499 (5'D) activity is not present in the liver of metamorphosing bullfrog tadpoles (Becker et al., 1997), T_4 and
500 T_3 would be metabolized by Pathway (I) and Pathway (II), respectively. From our study, it is unclear which
501 route in both pathways is predominant. The reactions that are catalyzed quickly and slowly, which are from
502 this study and Galton and Hiebert, (1987), are indicated by bold and narrow arrows, respectively. The
503 speed of the reactions that have not been determined are indicated by broken arrows.

Figure

Figure 1

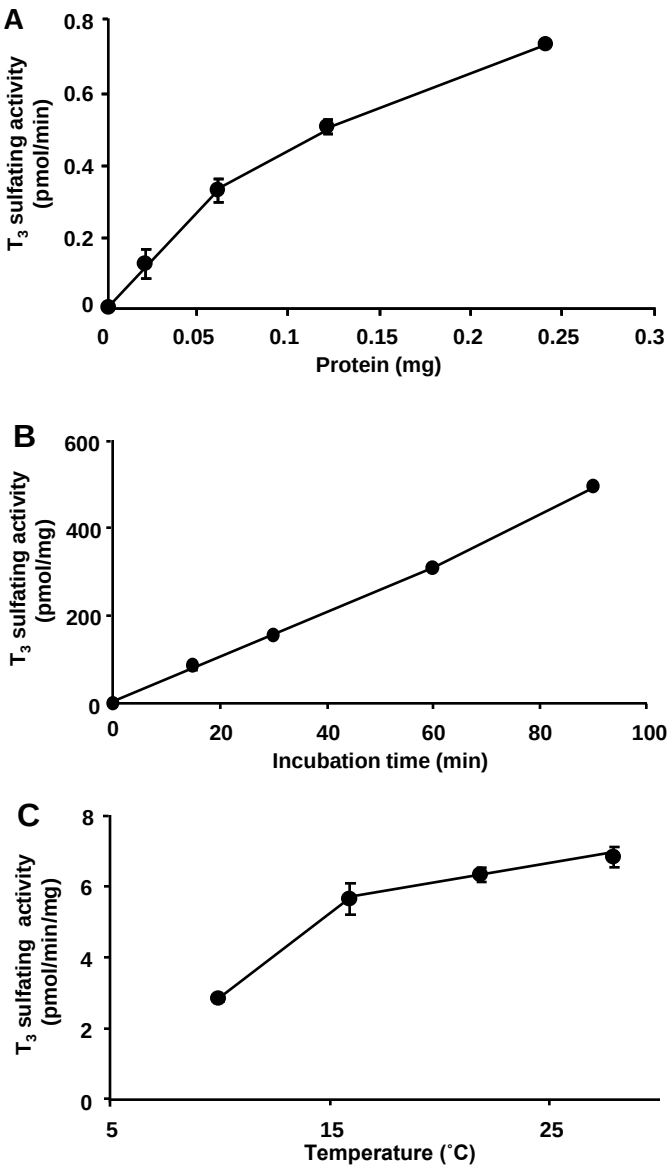
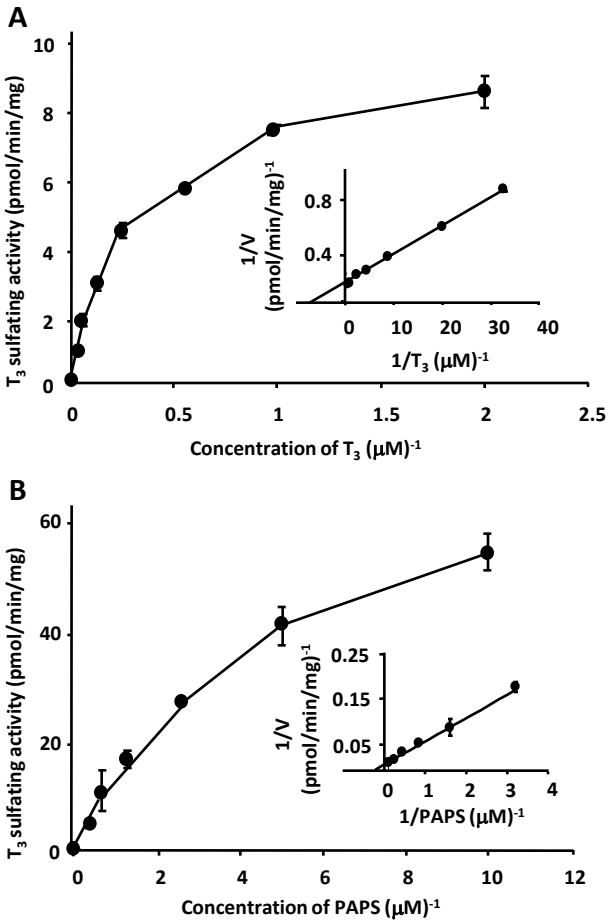
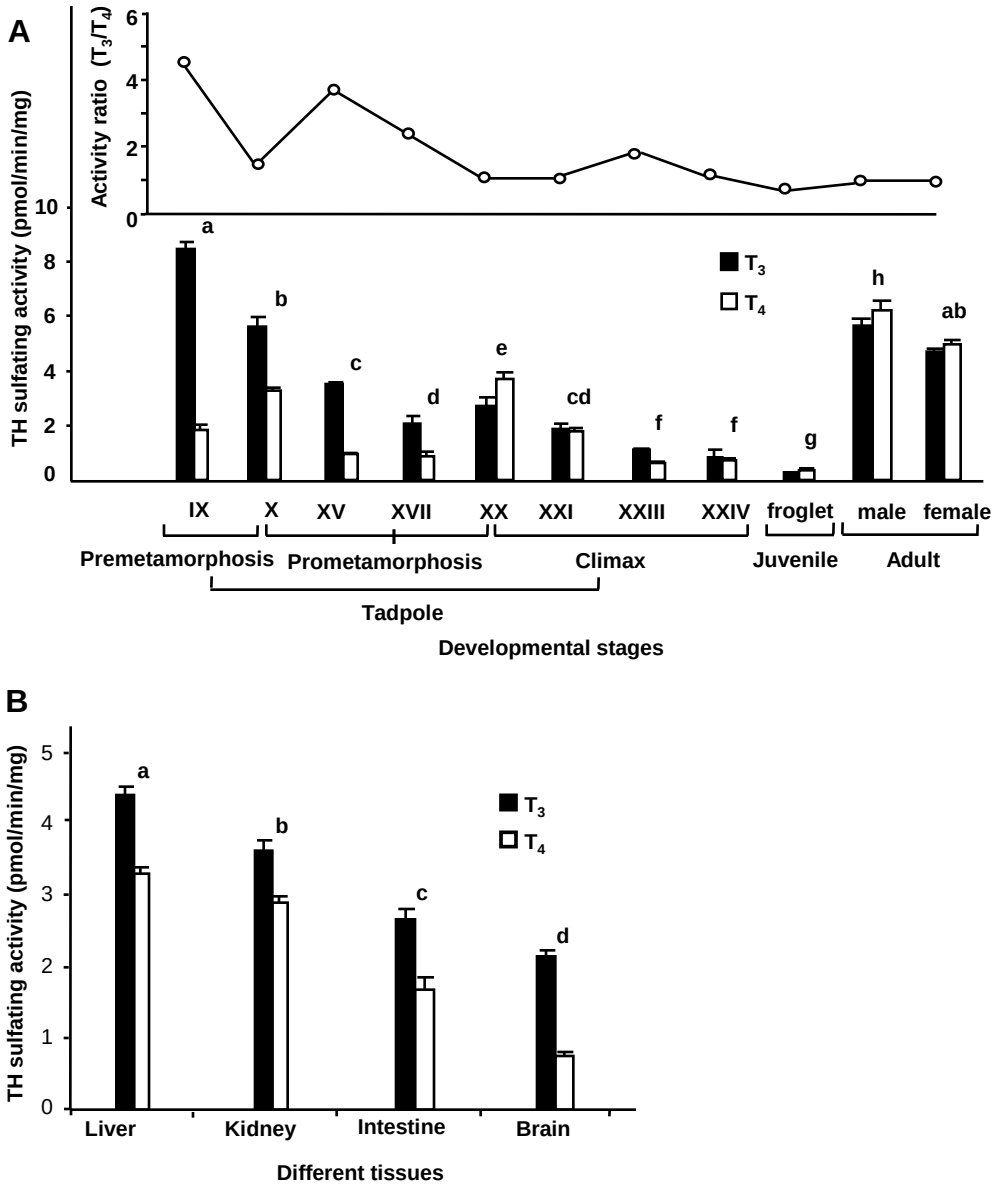


Figure 2


A
T3 sulfating activity (pmol/min/mg)
Concentration of T3 (μM)-1
1/V (pmol/min/mg)-1
1/T3 (μM)-1
B
T3 sulfating activity (pmol/min/mg)
Concentration of PAPS (μM)-1
1/V (pmol/min/mg)-1
1/PAPS (μM)-1

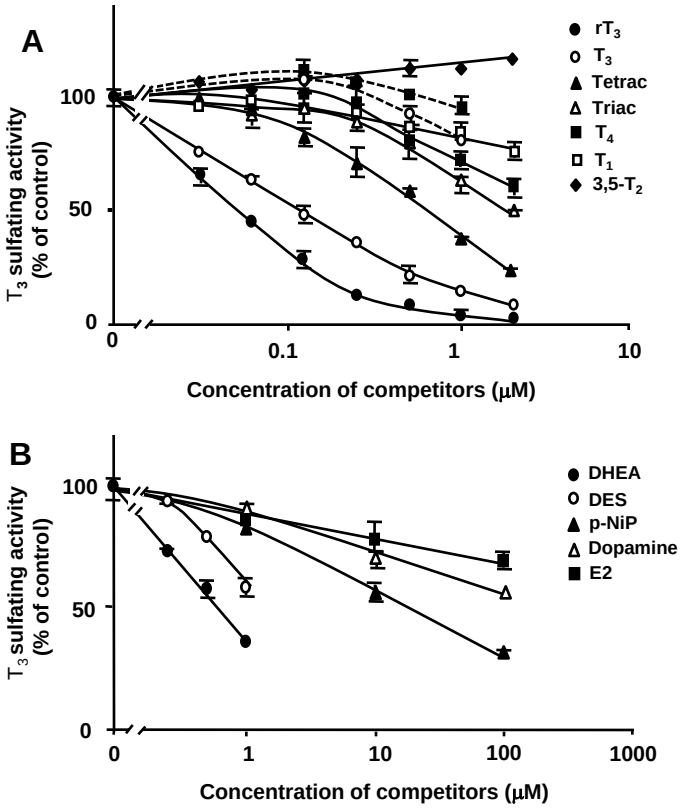
Figure

Figure 3



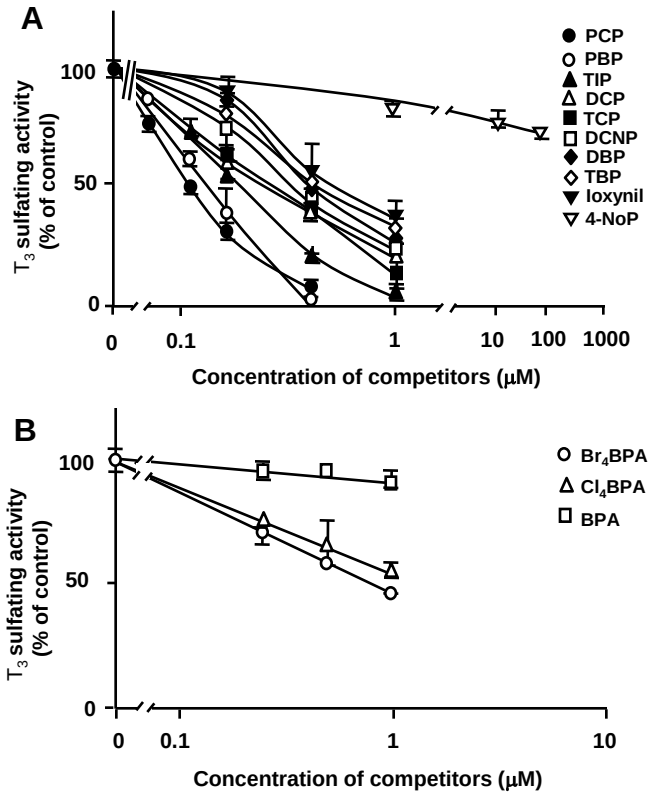
Figure

Figure 4



Figure

Figure 5



Figure

Figure 6