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Characterization of little skate (*Leucoraja erinacea*) recombinant transthyretin:
zinc-dependent 3,3',5-triiodo-L-thyronine binding

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Abbreviations: BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; HIU,
5-hydroxyisourate; HIUHase, 5-hydroxyisourate hydrolase; HPLC, high-performance liquid
chromatography; IC₅₀, 50% inhibitory concentration; ICP-OES, inductively coupled plasma optical
emission spectroscopy; IPTG, isopropyl β-D-1-thiogalactopyranoside; K_d, dissociation constant; MBC,
maximun binding capacities; PAGE, polyacrylamide-gel electrophoresis; reverse T3 or rT3,
3,3',5'-triiodo-L-thyronine; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; T3,
3,3',5-triiodo-L-thyronine; T4, L-thyroxine; TH, thyroid hormone; TTR, transthyretin.

Abstract

Transthyretin (TTR) diverged from an ancestral 5-hydroxyisourate hydrolase (HIUHase) by gene duplication at some early stage of chordate evolution. To clarify how TTR had participated in the thyroid system as an extracellular thyroid hormone (TH) binding protein, TH binding properties of recombinant little skate *Leucoraja erinacea* TTR was investigated. At the amino acid level, skate TTR showed 37–46% identities with the other vertebrate TTRs. Because the skate TTR had a unique histidine-rich segment in the N-terminal region, it could be purified by Ni-affinity chromatography. The skate TTR was a 46-kDa homotetramer of 14.5 kDa subunits, and had one order of magnitude higher affinity for 3,3',5-triiodo-L-thyronine (T3) and some halogenated phenols than for L-thyroxine. However, the skate TTR had no HIUHase activity. Ethylenediaminetetraacetic acid (EDTA) treatment inhibited [125 I]T3 binding activity whereas the addition of Zn^{2+} to the EDTA-treated TTR recovered [125 I]T3 binding activity in a Zn^{2+} concentration-dependent manner. Scatchard analysis revealed the presence of two classes of binding site for T3, with dissociation constants of 0.24 and 17 nM. However, the high-affinity sites were completely abolished with 1 mM EDTA, whereas the remaining low-affinity sites decreased binding capacity. The number of zinc per TTR was quantified to be 4.5–6.3. Our results suggest that skate TTR has tight Zn^{2+} -binding sites, which are essential for T3 binding to at least the high-affinity sites. Zn^{2+} binding to the N-terminal histidine-rich segment may play an important role in acquisition or reinforcement of TH binding ability during early evolution of TTR.

Keywords: transthyretin, thyroid hormone, divalent cation, endocrine disruption, 5-hydroxyisourate hydrolase, *Leucoraja erinacea*

1. Introduction

Plasma thyroid hormone (TH) binding proteins ensure the adequate distribution of THs in circulation to tissues. Plasma TH-binding proteins consist of thyroxine-binding globulin, transthyretin (TTR) and albumin (Robbins, 1996), all of which appeared during vertebrate evolution. However, the phylogenetic distribution of these TH-binding proteins is species-specific. TTR acts as a major TH-binding protein in some fish (Santos and Power, 1999; Yamauchi et al., 1999). In addition, lipoproteins and albumin are also known as major TH-binding proteins with low affinity for THs in immature salmonids (Cyr and Eales, 1992; Richardson et al., 2005). However, there is a lack of information about the TH binding properties of TTR from lower vertebrates such as elasmobranchs.

Chondrichthyes or cartilaginous fishes are an extant group that diverged from the earliest gnathostomes about 395 million years ago (Benton, 2005). Most elasmobranchs, including skates, have important ecological positions as top- or meso-predators within marine food webs. However, little is known about the non-natural ligands of skate TTR, including agricultural, industrial, and pharmaceutical chemicals that may be expected to displace THs from TTR and act as endocrine disruptors.

The TTR gene arose from an ancestral 5-hydroxyisourate hydrolase (HIUHase) gene by neo-functionalization after gene duplication at some early stage of chordate evolution (Ramazzina et al., 2006; Zanotti et al., 2006). Because this event occurred around the time that the thyroid system was established, the ancestor of TTR is likely to have acquired the ability to bind TH soon after gene duplication. Intriguingly, only two point mutations in zebrafish or amphioxus HIUHases resulted in the acquisition of TH binding activity and the loss of 5-hydroxyisourate (HIU) hydrolysis activity (Cendron et al., 2011; Li et al. 2013).

The N-terminal region of TTR forms a random-coiled structure with high conformational flexibility (Hamilton et al., 1993), and has important roles in the specificity and affinity of TH binding (Prapunpoj et al., 2006). It has been proposed that the N-terminal region has evolved from a longer and more hydrophobic region in lower vertebrates to a shorter and more hydrophilic region in eutherians, by unidirectional changes (Aldred et al., 1997; Prapunpoj et al., 2000), with functional changes in TH-binding specificity from for 3,3',5-triiodo-L-thyronine (T3) to for L-thyroxine (T4) (Yamauchi et al., 1993; Chang et al., 1999). Through the survey of several expressed sequence tag and genome databases of elasmobranch

fishes, we found TTR cDNA in the little skate *Leucoraja erinacea* database (<http://skatebase.org>), and noticed that such unidirectional changes may not be true for the skate TTR. Because the skate TTR has a highly hydrophilic stretch containing four histidine residues in the N-terminal region as if the skate TTR has a histidine tag. From this unique structure we supposed that functional analyses of the TTR gene superfamily in primitive gnathostomes may provide a good model for understanding the processes of neo-functionalization found in duplicated genes.

As a first step towards elucidating how TTR has participated in the thyroid system as a TH-binding protein during vertebrate evolution, we investigated TH binding properties and HIUHase activity of the recombinant skate TTR.

2. Materials and Methods

2.1. Reagents

³,^{3'}[¹²⁵I],5-Triiodo-L-thyronine (81 TBq/mmol, carrier free) was purchased from PerkinElmer (Waltham, MA, USA). 3,3',5-Triiodo-L-thyronine, T4, D-T3, 3,3',5'-triiodo-L-thyronine (reverse T3 or rT3), 3,3',5-triiodothyroacetic acid, 3,3',5,5'-tetraiodothyroacetic acid, diiodo-L-tyrosine, monoiodo-L-tyrosine, benzo[*a*]pyrene and pentachlorophenol were obtained from Sigma-Aldrich (St. Louis, MO, USA). Bisphenol A, 2,4,6-triiodophenol, 2,4,6-tribromophenol, 2,4,6-trichlorophenol, pentabromophenol, *o*-*t*-butylphenol, mirex, malathion (diethyl 2-dimethoxyphosphinothioylsulfanylbutanedioate, analytical standard), benzophenone, *n*-butylbenzyl phthalate, bis(2-ethylhexyl) adipate, bis(2-ethylhexyl) phthalate, dicyclohexyl phthalate, di-*n*-butyl phthalate, tributyltin (IV) chloride, α -benzoepin (α -endosulfan; 6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9,10-methano-2,4,3-benzodithiepine), dicofol (Kelthane, 2,2,2-trichloro-1,1-bis(4-chloro-phenyl)ethanol, analytical standard), urea, *Candida* sp. uricase and trimethylamine N-oxide were purchased from Wako Pure Chemical Industries (Osaka, Japan). 3,3',5,5'-Tetrabromobisphenol A, 3,3',5,5'-tetrachlorobisphenol A and 3,5-diiodo-L-thyronine were from Tokyo Chemical Industry (Tokyo, Japan). 2,4-Dichlorophenoxyacetic acid, 4-nonylphenol, di-2-ethyl phthalate and 2,4-dinitrophenol were purchased from Kanto Chemicals (Tokyo, Japan). Isopropyl

β -D-1-thiogalactopyranoside (IPTG) was obtained from Nacalai Tesque (Kyoto, Japan). 2-Isopropylphenol was from Lancaster. Methoprene (isopropyl (E,E')-(RS)-11-methoxy-3,7,11-trimethyldodeca-2,4-dienoate) was from Ehrenstorfer Quality (Augsburg, Germany). Ioxynil (3,5-diiodo-4-hydroxybenzonitril, analytical standard) and acetochlor (2-chloro-N-(ethoxymethyl)-N-(2-ethyl-6-methylphenyl)acetamide) were obtained from Riedel-de Haën (Seeize, Germany). Ni-resins were from Bio-Rad (Ni-IMAC Profinity, Hercules, CA, USA) and Invitrogen (ProBond Nickel-Chelating Resin, Carlsbad, CA, USA). Co-resin was obtained from Clontech (Talon Metal Affinity Resin, Mountain View, CA, USA). All other chemicals used in this study were either chromatography grade or the highest grade available and were purchased from Wako Pure Chemical Industries or Nacalai Tesque.

All chemicals tested as endocrine disrupting chemicals were dissolved in dimethylsulfoxide or 0.5 N NaOH to concentrations of 2–5 mM. These chemicals were then diluted with an appropriate buffer. For TTR pull-down assay, a blank assay without proteins with solvent alone at less than 1.0% (v/v) was done. The solvent did not affect the recovery of TTR in the pull-down assay. For competitive [¹²⁵I]T3 binding assay, a control assay without test chemicals was performed in the presence of the solvent alone and at less than 0.4% (v/v). The solvent did not affect the competitive [¹²⁵I]T3 binding assay.

2.2. Protein expression and purification

The skate TTR cDNA (accession no. CV221819, 607 bp) was amplified by polymerase chain reaction (PCR) with sense primer 5'-GTTCATATGCCACATAGTCACGGCGACC-3' (64–83) with a recognition site for *NdeI* (underlined), and antisense primer 5'-GTAGGATCCGGTTTTTCACGAGGTGTTAAG-3' (468–449) with a recognition site for *BamHI* (underlined). PCR was carried out in 50 μ L of solution containing 0.2 mM dNTP, 0.2 μ M of each primer, 1 ng template DNA and 1.25 U DNA polymerase (PrimeSTAR HS, TAKARA, Siga, Japan), using the following protocol: 98°C (15 s), and 20 cycles of 98°C (10 s), 65°C (5 s) and 72°C (30 s), followed by 72°C (5 min). Amplicons (64–449) were cloned between the *NdeI* and *BamHI* sites of a pET3a expression vector (Novagen, Madison, WI). Plasmids for expression were transformed into *Escherichia coli* Rosetta 2(DE3)pLysS (Novagen). Bacteria were usually grown in 50 mL LB medium with 50 μ g/mL ampicillin and 34 μ g/mL chloramphenicol at 37°C until the absorbance at 600 nm reached 0.8. The temperature was lowered to 18°C, 0.1 mM IPTG was added, and

incubation was continued for 16 h. Bacteria were pelleted by centrifugation ($1,200 \times g$) and stored at -35°C until used.

After suspending the bacterial pellet with 5 mL of homogenization buffer (1 mM imidazole, 0.3 M NaCl, 50 mM sodium phosphate, pH 8.0, 1 mM benzamidine hydrochloride, and 1 mM phenylmethylsulfonyl fluoride), the cells were disrupted by sonication 20 times for 10 s on ice at the range of 5 (UltraS homogenizer, VP-30S, TAITEC, Saitama, Japan), and the lysate was centrifuged at $18,000 \times g$ for 15 min at 4°C . Recombinant proteins were isolated from the other proteins in the supernatant by nickel or cobalt affinity chromatography (0.5 mL of the resin), by stepwise elution with various concentrations of imidazole (5, 20, 60, 150, 250 and 500 mM) in 0.3 M NaCl and 50 mM sodium phosphate, pH 8.0. Peak fractions were immediately applied to a Cellulofine GCL-2000 sf column (1.5×93 cm, Seikagaku Co., Tokyo, Japan), which had been equilibrated with 20 mM Tris, pH 7.5. Purified protein was stored in 10% glycerol at -85°C for later use. The yields of the recombinant proteins were 0.6–0.8 mg/50 mL bacterial culture. The recombinant rainbow trout HIUHase with three histidine residues at the N-terminal end and the recombinant skate TTR were also purified by ammonium sulfate precipitation, hydrophobic interaction chromatography on Phenyl Cellulofine column (1.0×10 cm, Seikagaku Co.) and then gel filtration chromatography on Cellulofine GCL-2000 sf column, as described previously (Kasai et al., 2013).

2.3. Protein analyses

Protein concentration was measured by the micro-Lowry method (Jain et al., 2002) with bovine serum albumin (BSA) as the standard. Proteins were electrophoresed on an sodium dodecyl sulfate (SDS)-polyacrylamide gel (15%) (Laemmli, 1970) with molecular markers: phosphorylase b (97 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α -lactalbumin (14.4 kDa). The gel was stained and proteins were visualized with Coomassie Brilliant Blue.

The molecular mass of TTR was estimated by high-performance liquid chromatography (HPLC) on YMC-Pack Diol 120 (500×8.0 mm, YMC, Kyoto, Japan) and YMC-Guardpack Diol-12 (30×8.0 mm), which were pre-equilibrated and eluted with 50 mM Tris-HCl, pH 7.5, and 140 mM NaCl, at flow rate of 1 mL/min at 25°C .

2.4. Ligand binding assay using a metal chelate affinity resin (TTR pull-down assay)

Iodothyronine or chemical binding was carried out by the TTR pull-down assay using a metal chelate affinity resin (Kasai et al., 2013). Briefly, TTR (0.5 μ M) was incubated with iodothyronines or chemicals (0.5 μ M) in 500 μ L of Tris-buffered saline, pH 7.5, for 1 h at 4°C, and the mixture was further incubated with Co-resin (5 μ L bed volume) for 1 h at 4°C in a 600- μ L plastic tube using a rotator. After a brief centrifugation, the TTR-ligand complex bound to the resin in the pellet was immediately recovered by the addition of 20 μ L of elution buffer (500 mM imidazole, 0.3 M NaCl, and 50 mM sodium phosphate, pH 8.0). The TTR-ligand complex released from the resin was mixed with the same volume of 1% acetic acid in methanol to extract the ligand from the complex. The mixture was agitated then centrifuged at $12,000 \times g$ for 10 min at 4°C. The ligand (15 μ L) in the supernatant was analyzed by reverse phase-HPLC on a C₁₈ analytical column (Mightysil RP-18 GP, 250 mm $\times$ 4.6 mm, 5 mm particle diameter, Kanto Chemical, Tokyo, Japan) with a UV detector at 254 nm, as described previously (Kasai et al., 2013). The amount of the ligand was quantified by comparison with the standards (4–100 pmol), with the lower limits of quantitation for iodothyronines and environmental chemicals of 2.0–7.2 pmol and 6–14.2 pmol, respectively, which were three times higher than the lower limits of detection.

2.5. [¹²⁵I]T3 binding assay

Unlike bony fishes, cartilaginous fishes retain a high concentration of organic osmolytes, such as urea (300–400 mM), in their body fluids to maintain a high plasma osmolality that is similar to that in the external environment. To counteract destabilization of proteins by urea, cartilaginous fishes also have methylamines, e.g., trimethylamine N-oxide (Yancey, 2005). Recombinant TTR (7.5 ng/tube, 0.53 nM) was incubated with 0.1 nM [¹²⁵I]T3 in 250 μ L of buffer (20 mM Tris, pH 7.5, 280 mM NaCl, 360 mM urea, and 90 mM trimethylamine N-oxide) in the presence or absence of test compounds (cations, unlabeled iodothyronines or environmental chemicals) for 1.0 h at 4°C, unless otherwise noted. In some cases, various concentrations of TTR were pre-incubated with 1 mM ethylenediaminetetraacetic acid (EDTA). After dilution with the buffer, TTR (0.53 nM) was used for [¹²⁵I]T3 binding assay in the presence or absence of cations. For the Scatchard plot of T3 binding to both high-affinity and low-affinity sites, skate TTR was incubated with 0.1 nM [¹²⁵I]T3 in the presence or absence of increasing concentrations of

unlabeled T3. The dissociation constant (K_d) value for high affinity sites was expected to be 10^{-10} M from this experiment. To estimate more accurately the K_d value, TTR was incubated with [125 I]T3 (0.052 to 0.4 nM) in the presence or absence of 5 μ M of unlabeled T3. [125 I]T3 bound to proteins was separated from free [125 I]T3 by the polyethyleneglycol method (Yamauchi et al., 1993). The radioactivity of the samples was measured in a gamma counter (2480 WIZARD²; PerkinElmer). The amount of [125 I]T3 bound non-specifically was derived from the radioactivity of samples incubated with 5 μ M unlabeled T3. The non-specific binding value was subtracted from the amount of total bound [125 I]T3 that was derived from the radioactivity of samples incubated without unlabeled T3, to give the value of specifically bound [125 I]T3. The K_d s and maximum binding capacities (MBCs) for T3 binding were determined from Scatchard plots for a single class (Scatchard, 1949) or for two classes (Rosenthal, 1967).

2.6. Determination of the average number of divalent cations per TTR

TTR samples were prepared by Co-affinity chromatography and gel filtration chromatography on Cellulofine GCL 2000-sf column. Alternatively, to avoid the effects of Co-resin on the metal contents of TTR, TTR samples were also prepared by ammonium sulfate precipitation, hydrophobic interaction column chromatography, and gel filtration column chromatography, as shown previously (Kasai et al., 2013). The content of divalent cations in TTR samples (1.5 mL in 4 mM Tris, pH 7.5, 0.16–0.65 mg/mL) was quantified three times by inductively coupled plasma optical emission spectroscopy (ICP-OES) (Optima 3300 DV, PerkinElmer) using calibration curves of zinc, copper, nickel, cobalt and iron standards (0, 0.01, 0.05, 0.1, 0.5, 1.0, 5.0 mg/L in 0.1 mol/L HNO₃) (Wako). The lower limits of quantitation were 0.011 mg/mL, and the lower limits of detection were 0.002 mg/mL.

2.7. HIU hydrolysis assay

HIU hydrolysis assay was conducted according to the previous method (Kasai et al., 2013). Enzyme activity was measured at 25–27°C by following the decrease in absorbance at 312 nm as HIU was hydrolyzed (Lee et al., 2005). 5-Hydroxyisourate was generated *in situ* by the addition of 50 μ L of 0.32 μ M uricase in 50 mM potassium phosphate buffer, pH 7.5, to 400 μ L of 100 μ M uric acid in the same buffer. When the amount of HIU reached a maximum (2.4 min after starting the reaction), 10 μ L of HIUHase (2

μg) or TTR (2 μg) was added to the mixture in a microcuvette. Absorbance at 312 nm was immediately monitored at 0.2 min intervals using a spectrophotometer (U-3210, Hitachi, Japan). The control assay was carried out similarly except that HIUHase or TTR solution was replaced by phosphate buffer. As HIU is not stable, changes in absorbance at 312 nm does not reflect correctly the enzymatic hydrolysis of HIU. Therefore, the enzyme activity was expressed as the difference in absorbance at 312 nm between the presence (spontaneous + enzymatic hydrolysis of HIU) and the absence (spontaneous hydrolysis of HIU) of proteins.

2.8. Statistics

The data are the mean ± standard error of the mean ($n \geq 3$), unless otherwise noted. Differences between groups were analyzed by a one-way analysis of variance, with the Scheffe's test for multiple comparisons. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Comparison of TTR amino acid sequences

The skate TTR amino acid sequence shared 37–46% identities with the other vertebrate TTR amino acid sequences shown in Fig. 1, although the skate TTR amino acid sequences shared only 24–27% identities with the amphioxus and trout HIUHase amino acid sequences (data not shown). Significant variations of amino acid residues were detected at the N-terminal regions, where the skate and lamprey TTR had a histidine-rich segment of high hydrophilicity, HSHGDHH and HKSHESH, respectively. Thirty residues were invariant in the amino acid sequences among the 9 TTR sequences.

3.2. Molecular features of recombinant skate TTR proteins

Skate TTR could be purified by nickel-affinity chromatography (Fig. 2A) and gel filtration chromatography. Skate TTR molecule may be a slightly compact tetramer. The molecular mass of the skate TTR under native conditions was estimated to be ~46 kDa by gel filtration column chromatography (Fig. 2B). This value was somewhat smaller than the molecular mass of the tetramer (56.8 kDa) estimated from

the amino acid sequence. The molecular mass for the TTR polypeptide was 14.5 kDa on SDS-polyacrylamide-gel electrophoresis (PAGE) (Fig. 2C).

3.3. Binding of iodothyronines and environmental chemicals to recombinant skate TTR using TTR pull-down assay

3,3',5-Triiodo-L-thyronine bound to skate TTR then extracted with methanol was eluted at the same retention time (11.46 min) on reverse phase-HPLC as the T3 standard, as shown previously (Kasai et al., 2013). The peak of T3 was negligible when the same pull-down assay was done without TTR (blank). Skate TTR exhibited high binding activity for natural ligands T3 and T4, and related compounds, with the following rank order affinity: D-T3 $\geq$ 3,3',5,5'-tetraiodothyroacetic acid, T3, T4 and 3,3',5-triiodothyroacetic acid $\geq$ reverse T3 > 3,5-diiodo-L-thyronine (Fig. 3A). With regard to environmental chemicals (Fig. 3B), skate TTR showed binding activity for triiodophenol and ioxynil, but not for benzophenone and 2,4-dinitrophenol.

3.4. [¹²⁵I]T3 binding activity of recombinant skate TTRs

3.4.1. Effects of divalent cations

To test the possibility that the skate TTR has significant affinity for metal ions, [¹²⁵I]T3 binding was performed in the presence of 1 mM divalent cations. However, any cations tested did not show statistically significant effects on [¹²⁵I]T3 binding (Fig. 4A). We next pre-incubated skate TTR (0.53–2640 nM) with EDTA (1 mM) at various molar ratios followed by [¹²⁵I]T3 binding assay at 0.53 nM of TTR, to determine the most effective molar ratio of TTR/EDTA for eliciting the chelating action of EDTA (Fig. 4B). [¹²⁵I]T3 binding was decreased by more than a half at the TTR pre-incubation concentration of 264 nM (TTR/EDTA molar ratio of 1/3,800), and by about 90% at the TTR pre-incubation concentration of 26.4 nM (TTR/EDTA molar ratio of 1/38,000). Using the TTR pre-treated with 1 mM EDTA at the TTR/EDTA molar ratio of 1/38,000, the effects of divalent cations (1 mM) on [¹²⁵I]T3 binding to TTR (0.53 nM) was investigated (Fig. 4C). Zn²⁺, Ni²⁺ and Mn²⁺ (each 1 mM) recovered [¹²⁵I]T3 binding activity up to 60–90% of that of the TTR not pre-treated with EDTA. Ca²⁺ and Mg²⁺ had little effect or were not effective. The effects of Fe²⁺, Co²⁺, and Cu²⁺ on [¹²⁵I]T3 binding could not be determined, because of the formation

of insoluble materials under our assay conditions (data not shown). Addition of 20 μM Zn^{2+} activated partially [^{125}I]T₃ binding in spite of the presence of the residual EDTA (final 20 μM ; Fig 4D). [^{125}I]T₃ binding activity reached a plateau at more than 50 μM Zn^{2+} .

3.4.2. Competitive inhibition of [^{125}I]T₃ binding to recombinant skate TTR by iodothyronines and other compounds

[^{125}I]T₃ binding was investigated in the presence of various concentrations of iodothyronines and their related compounds (Fig. 5). The most powerful competitor was 3,3',5-triiodothyroacetic acid with 50% inhibitory concentration (IC₅₀) of 1.0 ± 0.2 nM, which was two times less than the IC₅₀ for T₃ (Table 1). The rank order binding affinity was 3,3',5-triiodothyroacetic acid $\geq$ T₃ $\geq$ D-T₃ $\geq$ 3,3',5,5'-tetraiodothyroacetic acid > T₄ > reverse T₃ > 3,5-diiodo-L-thyronine > diiodo-L-tyrosine and moniodo-L-tyrosine. T₃ was 13 times more potent than T₄. Acetic acid analogs (3,3',5-triiodothyroacetic acid and 3,3',5,5'-tetraiodothyroacetic acid) were 2-3 times more potent than the corresponding iodothyronines (T₃ and T₄). Iodotyrosines had no affinity for skate TTR.

As a primary screening of environmental chemicals that interfere with [^{125}I]T₃ binding to skate TTR in vitro, inhibitory effects of chemicals on [^{125}I]T₃ binding was investigated at 1 μM concentration. Out of 29 chemicals tested, 8 chemicals, all of which were phenolic compounds consisting of one or two phenolic rings with several halogen atoms, strongly inhibited [^{125}I]T₃ binding (Fig. 6A). Phenolic compounds without halogen atoms, phthalates and the other chemicals tested were not significant competitors, even if they had benzene or phenolic ring(s).

[^{125}I]T₃ binding in the presence of varying concentrations of the eight chemicals that strongly inhibited [^{125}I]T₃ binding was tested (Fig. 6B). The most effective inhibitor of [^{125}I]T₃ binding to skate TTR was pentabromophenol, with an IC₅₀ of 1.4 ± 0.3 nM, which was between those for 3,3',5-triiodothyroacetic acid and T₃ (Table 1). The rank order binding affinity was pentabromophenol $\geq$ triiodophenol $\geq$ pentachlorophenol $\geq$ 3,3',5,5'-tetrabromobisphenol A $\geq$ 3,3',5,5'-tetrachlorobisphenol A and ioxynil $\geq$ trichlorophenol and tribromophenol.

3.4.3. Kinetics of [^{125}I]T₃ binding to skate TTR

Scatchard plots revealed the presence of two classes of binding sites for T3 (Fig. 7), with K_{d1} and K_{d2} of 0.52 ± 0.03 nM and 17 ± 3 nM, and MBC_1 and MBC_2 of 2.2 ± 1.0 pmol/ μ g protein and 19 ± 5 pmol/ μ g protein, respectively. To determine more precisely the K_{d1} value, we conducted Scatchard analysis at lower concentrations and within a more narrow range of [125 I]T3, which showed the K_{d1} value of 0.24 ± 0.04 nM (Fig. 7 inset). In the presence of 1 mM EDTA, the Scatchard plot showed a single class of binding sites, where high-affinity sites were completely abolished. The K_d value was estimated to be 21 ± 1 nM, which nearly corresponded to that for the low-affinity sites of the TTR untreated with EDTA, with decreased MBC value, 1.4 ± 0.1 pmol/ μ g protein (Table 2).

3.5. HIU hydrolysis activity of recombinant skate TTR and recombinant rainbow trout HIUHase

As the production of HIU, a substrate for HIUHase, reached a peak at 2.4 min after the addition of uricase into the reaction mixture (Fig. 8), we investigated HIUHase activity from this time point by adding trout HIUHase or skate TTR. Hydrolysis of HIU by the trout HIUHase was clearly detected at the concentration of 4.4 μ g/mL. Activity (difference in absorbance at 312 nm between the samples in the absence and the presence of HIUHase) peaked at 2.4–4.0 min and decreased thereafter. However, there was no significant effect of the skate TTR on the hydrolysis of HIU at the same concentration (Fig. 8).

3.6. Contents of divalent cations in skate TTR molecule

The zinc content measured by using ICP-OES was 4.47–6.25 mol/mol TTR (Table 3). Additionally, nickel and copper were also detected at 0.40 and 0.36–2.29 mol/mol TTR, respectively. The contents of cobalt and iron were below detectable levels. The TTRs purified using Co-resin and hydrophobic interaction chromatography had similar total metal contents, 6.76–7.01 mol/mol TTR. The same fractions on gel chromatography of the cell extract, which were obtained from *E. coli* harboring intact pET3a, did not contain detectable levels of the metals analyzed.

4. Discussion

The present study demonstrates that skate TTR is a zinc-dependent TH-binding protein. This feature is

supported by the presence of a histidine-rich segment in the N-terminal region, the ability to be purified by metal chelate affinity chromatography, inactivation of T3 binding by EDTA and recovery of T3 binding activity by divalent cations. Skate TTR had high-affinity and low-affinity sites for T3 with K_{ds} of 0.24 nM and 17 nM, but not HIU hydrolysis activity. All of the high affinity and most of the low affinity sites for T3 were abolished by the treatment of EDTA. Analysis by IPC-OES revealed that the skate TTR molecule contains 4.47–6.25 zinc ions. Therefore, zinc ion may be essential for the skate TTR to bind T3.

We discovered that a histidine-rich segment, HSHGDHH, located in the N-terminal region of skate TTR may act as a natural histidine tag, which facilitated the purification of this protein by Ni-affinity chromatography and may also provide a novel ligand binding assay (TTR pull-down assay) for investigating the ligand interaction. The histidine-rich segments of the skate and lamprey TTRs resemble the zinc-binding motif of metalloproteases (HEXXH or HEXXHXXGXXH/D) (Cerdà-Costa and Gomis-Rüth 2014), but are quite different from the metal-binding motif of metallothionein with cysteine-rich motifs (Vašák and Meloni, 2011). TTR is a homotetramer consisting of a dimer of dimers (subunits A and B, and subunits C and D). As the N-terminal regions of two subunits (A and D, or B and C) are located in the vicinity of the entrance of either hormone binding pocket (Hamilton et al., 1993), the two histidine-rich segments in the N-terminal regions are highly likely to act as a histidine tag comparable with the ordinary 6× histidine-tag. In this study, we do not exclude the possibility of the presence of several binding sites for divalent cations in skate TTR molecule besides the N-terminal region.

Divalent cation binding to skate TTR is necessary to elicit strong T3 binding activity that may occur at physiological conditions, in which Zn^{2+} is the best possible candidate cation. [^{125}I]T3 binding to skate TTR was inactivated by EDTA and re-activated by divalent cations in vitro. When skate TTR (264 nM) was pre-treated with EDTA (1 mM) at the molar ratio of 1/3,800, [^{125}I]T3 binding to the skate TTR was partially inhibited. This suggests that most of the zinc ions involved in T3 binding do not dissociate from the TTR of the micromolar range. The concentration of Zn^{2+} that was needed to reactivate the [^{125}I]T3 binding activity, ~0.05 mM, was less than the physiological range (0.1–0.2 mM) of Zn^{2+} in fish plasma (Sturrock et al. 2013). Scatchard analysis revealed the presence of two classes of binding sites. [^{125}I]T3 binding to all of the high affinity sites and most of the low affinity sites were lost with the addition of 1 mM EDTA. This finding leads us to the possibilities that Zn^{2+} binding is essential for skate TTR to express

at least high affinity sites for T3 binding and that Zn^{2+} at the concentration in plasma may modulate T3 binding to skate TTR in vivo. In this study we determined the average number of metal ions per TTR. If skate TTR samples consist of a mixture of the TTR components with different content of Zn^{2+} or the Zn^{2+} content of skate TTR in circulation differs locally, a variety of the number of Zn^{2+} per TTR may increase the functional diversity in T3 binding activity. Future study will need to address the physiological meanings of Zn^{2+} binding to TTR on the skate thyroid system.

This is the first report showing the divalent-dependent TH binding activity of TTR, although there are several reports regarding the effects of metal ions on the other functions of human TTR. Zn^{2+} binding decreases monomer stability to enhance TTR aggregation at an acidic pH (Palmieri et al., 2010) or amyloid formation of a mutant TTR (Wilkinson-White and Easterbrook-Smith, 2007), and decreases the affinity of TTR for RBP4 (Palmieri et al., 2010). Zn^{2+} is also essential for expressing the metalloprotease activity of human TTR, which was inhibited by EDTA or 1,10-phenanthroline and reactivated by divalent cations (Liz et al., 2012). Zinc content in human TTR was ~0.2 per TTR molecule (Liz et al., 2012), which was one order less than that in the skate TTR (4.47–6.25 per TTR). In addition, the skate TTR contained copper at 0.36–2.29 per TTR, and nickel at 0.4 per TTR. This means that four fifths of human TTR is independent of zinc-dependent functions. However, in the skate TTR, because of the high content of zinc, T3 binding activity which is highly zinc-dependent may be fully affected by the zinc content. Therefore, the deficiency of metal ions such as zinc may influence the functions of TTR to greater extent in skates compared with humans.

Zn^{2+} binding may be an ancient property of the TTR/HIUHase superfamily with high diversity. In the TTR family, Zn^{2+} binding is experimentally shown so far in the skate and human protein alone. Among the four Zn^{2+} binding sites (ZBS1 to ZBS4) proposed in human TTR (Palmieri et al., 2010), only ZBS1 (C10 and H56 in Fig. 1) is conserved in the vertebrate TTRs investigated. Among ten amino acid residues that constitute the four Zn^{2+} binding sites in the human TTR, four are identical and one is similar to the corresponding amino acid residues in the skate TTR. The histidine-rich segments found in the skate and lamprey TTRs are not detected in the bony fish and tetrapod TTRs (Fig.1) or HIUHases of various vertebrates. However, in the HIUHase family, our previous study (Kasai et al., 2013) showed that Zn^{2+} (50 μM) inhibits significantly the HIU hydrolysis activity of rainbow trout HIUHase. The protein structure of *E.*

coli HIUHase revealed the presence of zinc ions bound to the functional sites (Lundberg et al., 2006). All of these circumstances suggest that Zn²⁺ binding may be a common property of the HIUHase/TTR superfamily. Interestingly, the number of histidine residues in TTR polypeptide has changed during vertebrate evolution: 11 for lamprey and skate, 7–8 for bony fishes, 6 for amphibians, 5 for reptiles and a bird and 4 for humans (Fig. 1). It is likely that TTR has evolved from a strong to weak divalent cation binder (Wang et al., 2013). Although there is no experimental data to explain why the histidine content in TTR sequences is high in lamprey and skate TTRs and continuously decreased during vertebrate evolution, some of these histidine residues may participate in divalent cation binding and contribute to the acquisition or reinforcement of TH binding activity in an ancestor of the agnathan and cartilaginous fish TTRs soon after TTR diverged from ancestral HIUHase.

Skate TTR showed the highest affinity for T3 among the vertebrate TTRs studied so far, with K_{d1} of 10^{-10} M, which is comparable to those of nuclear TH receptors ($K_d=10^{-11}$ – 10^{-10} nM) (Oppenheimer et al., 1996). Giving that plasma TH levels are 10^{-9} – 10^{-8} M in several elasmobranchs: the whitetip reef shark *Triaenodon obesus* (Crow et al., 1999), dogfish *Squalus acanthias* (Leary et al., 1999) and Atlantic stingray *Dasyatis Sabina* (Volkoff et al., 1999), skate TTR may be functional as a T3-binder rather than T4-binder. The preference for T3 over T4 was also detected in bony fish (Kasai et al., 2013; Kawakami et al., 2006; Santos and Power, 1999; Yamauchi et al., 1999), amphibian (Prapunpoj et al., 2000; Yamauchi et al., 1993), reptilian (Prapunpoj et al., 2002) and bird (Chang et al., 1999), but not in mammalian TTRs (Chang et al., 1999), suggesting that TTR has originally evolved as a T3-binder during the early evolution of fish and changed as T4-binder during the early evolution of mammals (Richardson, 2007; 2015).

We applied the TTR pull-down assay for ligand binding to skate TTR. Ligand preference of TTR tested by this assay agreed well with that tested by the [¹²⁵I]T3 competitive binding assay for endogenous and exogenous ligands. The TTR pull-down assay is a convenient and a non-radioisotopic method that can estimate directly and semi-quantitatively the ligand-TTR interaction. Furthermore, the TTR-pull-down assay can detect several potent chemicals with different retention times on HPLC at the same time. The drawback is that relatively high concentrations of ligands and TTR are needed to quantify their amounts by HPLC, and that the accuracy of this assay is not high. Therefore, this assay may be suitable for the primary screening that allows to estimate semi-quantitatively environmental contaminants with significant affinity

for TTR and with significantly high absorption spectrum in the ultraviolet or visible region.

Skate TTR has significant high affinity for several halogenated phenolic compounds that structurally resemble THs (Howdeshell, 2002). Tetrabromobisphenol A and pentabromophenol were the most potent chemicals in halogenated phenolic compounds with two phenolic rings and with a single ring, respectively. The structural preference of ligand binding was well conserved in vertebrate evolution (Kudo et al., 2006; Meerts et al., 2000; Morgado et al., 2007; Yamauchi et al., 2003). Furthermore, in the structure-activity relationships, halogen species-dependency ($\text{Br}^- > \text{Cl}^-$ in pentahalogenated phenols; $\text{I}^- > \text{Br}^- \approx \text{Cl}^-$ in trihalogenated phenols; $\text{Br}^- > \text{Cl}^- > \text{no halogen}$ in bisphenol A) and halogen number-dependency (penta- > tri- in brominated and chlorinated phenols) are similar with previous studies using human (Meerts et al., 2000), chicken and amphibian TTRs (Kudo et al., 2006; Yamauchi et al., 2003).

Production of brominated phenols, used as a flame retardant or its intermediate, or as a wood preservative, may result in the release of brominated phenols to the aquatic environment for several decades (WHO, 2005). The annual consumption by humans of tetrabromobisphenol A is 89,400 tons in Asia, which is 56% of the total market demand in 2001 (Birnbaum and Staskal, 2004). As skates spend time in the sediments of the sea, where they feed on various invertebrates including decapod crustaceans, amphipods and polychaete worms, they are ecologically situated in the top or middle of the benthic food web. This ecological position may expose skates to hydrophobic and persistent organic pollutants retained in the benthic environments with a relatively high bioaccumulation. Therefore, investigating the inhibitory effects of persistent halogenated compounds on TH binding to skate TTR may be useful to assess the threat on the thyroid system from environmental chemicals in benthic environments.

In conclusion, we have demonstrated that skate TTR is a strong T3-binder in vitro, and that T3 binding to skate TTR is divalent cation-dependent and competitively inhibited by environmental phenolic compounds with halogens. The unique structure of a histidine-rich segment in the N-terminal region may act as a natural histidine tag, raising the possibility that the TH binding activity of the skate TTR may be modulated by Zn^{2+} in plasma. We proposed that a histidine-rich segment in the N-terminal region of TTR may play an important role in acquisition or reinforcement of TH binding activity via metal binding during early vertebrate evolution.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Figure legends

Fig. 1. Comparison of transthyretin (TTR) sequences with possible zinc-binding sites. Mature TTR amino acid sequences from lamprey (Manzon et al., 2007), skate (accession no. CV221819), trout (Yamauchi et al., 1999; accession.no.CX256523), seabream (Santos and Power, 1999), toad (Prapunpoj et al., 2000), bullfrog (Yamauchi et al., 1993, 1998), crocodile (Prapunpoj et al., 2002), chicken (Duan et al., 1991) and human (Kanda et al., 1974) were aligned. The N-terminal ends of the lamprey, skate and seabream TTRs are predicted, whereas those of the other TTRs are based on Edman degradation. The numbering of amino acid is based on that of human TTR. Features of secondary structure of human TTR (Hamilton et al., 1993) are indicated above the lamprey sequence. Histidine-rich segments in the disordered N-terminal region of the skate and lamprey TTRs are boxed. A segment that resembles a zinc-binding motif of metalloprotease (HEXXHXXGCCH/D) (Cerdà-Costa and Gomis-Rüth, 2014) is underlined. Four zinc-binding sites (ZBSs 1-4) proposed in human TTR (Palmieri et al., 2010) are also boxed: C10 and H56 for ZBS1, H88, H90 and E92 for ZBS2, H31, D74 and E72 (or E62 at acidic pH), H31, E61 and D74 for ZBS4. Marks (*) underneath the sequence alignment indicate positions where amino acid residues are invariant, among the 9 sequences. The numbers of histidine residues in the TTR sequences are shown on the right.

Fig. 2. Purification of recombinant skate transthyretin (TTR) by metal chelate affinity chromatography and gel chromatography. (A) SDS-PAGE of the fractions of metal chelate affinity chromatography. The recombinant skate TTR was separated from the other bacterial proteins in cell extract by metal chelate affinity chromatography on Ni-resin, and then eluted with the buffer containing 150 mM imidazole (*an arrow* in fraction *E4*). Lanes from left to right: *ppt*, pellet of cell lysate; *sup*, supernatant of cell lysate; *FT*, flow-through fraction; *W1* to *W4*, washing fractions; *E1* to *E6*, elution fractions with buffer containing 5, 20, 60, 150, 250, 500 mM imidazole, respectively. (B) High performance gel chromatography of purified recombinant skate TTR. Protein was applied to YMC Diol 120 High performance gel chromatography column (8 × 500 mm) in 50 mM Tris-HCl, pH 7.5, and 140 mM NaCl, at 1 mL/min. **Elution was monitored at absorbance at 280 nm.** Markers: Blue dextran (void volume, V_0); apoferritin (Fer, 440 kDa); alcohol dehydrogenase (ADH, 158 kDa); bovine serum albumin (BSA, 68 kDa); ovalbumin (OVA, 45 kDa); and myoglobin (Mb, 17 kDa). (C) SDS-PAGE of the peak fraction of Cellulofine GCL-2000 sf gel filtration

chromatography. *Arrow*, band of skate TTR.

Fig. 3. Ligand-binding to recombinant skate transthyretin (TTR) determined by the TTR pull-down assay using metal chelate affinity resin. After incubating TTR (14.2 μ g, 0.5 μ M) with each ligand (0.5 μ M) (*A*, iodothyronines and their acetic acid analogs; *B*, environmental chemicals) in 500 μ L of the incubation buffer, the ligand bound to TTR was analyzed by reverse phase-HPLC as described in the Materials and methods. The vertical axis of graphs indicates the amount of the bound ligand that was recovered in 40 μ L of the extraction buffer. Blank data obtained from samples in the absence of TTR were subtracted from those obtained from samples in the presence of TTR. Each value is the mean $\pm$ standard error of the mean ($n = 5-6$ for *panel A*; $n = 3$ for *panel B*). Different letters indicate significantly different means ($p < 0.01$). T3, 3,3',5-triiodo-L-thyronine; T4, L-thyroxine; Triac, 3,3',5-triiodothyroacetic acid; Tetrac, 3,3',5,5'-tetraiodothyroacetic acid; rT3, 3,3',5'-triiodo-L-thyronine, T2, 3,5-diiodo-L-thyronine, D-T3, 3,3',5-triiodo-D-thyronine, TIP, 2,4,6-triiodophenol, 2,4-DNP, 2,4-dinitrophenol.

Fig. 4. Effects of ethylenediaminetetraacetic acid (EDTA) and divalent cations on [125 I]T3 binding to recombinant skate transthyretin (TTR). (*A*) Effect of divalent cations of [125 I]T3 binding to skate TTR. TTR (0.53 nM) was incubated with 0.1 nM [125 I]T3 in the presence or absence (control) of 1 mM each divalent cation for 1 h at 4°C. (*B*) Effect of pre-treatment of TTR at various concentrations with 1 mM EDTA on [125 I]T3 binding. TTR (0.53–2640 nM) was pre-incubated with or without (control) 1 mM EDTA for 30 min for 4°C. After dilution 1, 5, 50, 500, or 5,000 times with the buffer, TTR (0.53 nM) was incubated with 0.1 nM [125 I]T3 for 1 h at 4°C. (*C*) Effects of divalent cations on [125 I]T3 binding to the TTR pre-treated with EDTA. TTR (26.4 nM) was pre-incubated with or without (control) 1 mM EDTA for 30 min for 4°C. After dilution 50 times with the buffer (final concentration of EDTA = 0.02 mM), TTR (0.53 nM) was incubated with 0.1 nM [125 I]T3 in the presence or absence of 1 mM of each cation for 1 h at 4°C. Binding activity of the TTR that was treated with neither EDTA nor cation (*EDTA*-, *cation* -) is indicated as the control (100%). (*D*). Zn^{2+} -dependent activation of [125 I]T3 binding to TTR. TTR (26.4 nM) was pre-incubated with 1 mM EDTA for 30 min for 4°C. After dilution 50 times with the buffer, TTR (0.53 nM) was incubated with 0.1 nM [125 I]T3 in various concentrations of Zn^{2+} for 1 h at 4°C. [125 I]T3 binding activity of the TTR that was

untreated with 1 mM EDTA (*open circle*) is indicated as control 100%. Each value is the mean $\pm$ standard error of the mean of triplicate determinations. Experiments were repeated two or three times. Asterisks indicate significant differences in T3 binding activity between the reference sample [cation (-) in *panel A*; EDTA (-) in *panel B*; EDTA(+) and cation (-) in *panels C and D*] and each test sample. *, $p < 0.05$; **, $p < 0.01$.

Fig. 5. Competition of iodothyronines and their related compounds for [125 I]T3 binding to recombinant skate transthyretin (TTR). TTR (0.53 nM) was incubated with 0.1 nM [125 I]T3 for 1 h at 4°C, in the presence or absence (control) of increasing concentrations of the following compounds: 3,3',5-triiodothyroacetic acid (Triac), T3, D-T3, 3,3',5,5'-tetraiodothyroacetic acid (Tetrac), T4, reverse T3 (rT3), 3,5-diiodo-L-thyronine (T2), 3,5-diiodo-L-tyrosine (Y2) and 3-iodo-L-tyrosine (Y1). Non-specific binding, which was 3–4 % of total binding, was subtracted from total binding to give values for specific binding. Each value is the mean of triplicate determinations. These experiments were repeated at least three times.

Fig. 6. Inhibition of [125 I]T₃ binding to recombinant skate transthyretin (TTR) by various environmental chemicals. (A) TTR (0.53 nM) was incubated with 0.1 nM [125 I]T3 in the presence of 1 μ M of each chemicals or solvent (dimethylsulfoxide, control) for 1 h at 4°C. Asterisks indicate significantly differences in [125 I]T₃ binding activity between control and test samples containing each environmental chemical (**, $p < 0.01$). (B) TTR (0.53 nM) was incubated with 0.1 nM [125 I]T3 for 1 h at 4°C, in the presence or absence (control) of increasing concentrations of the following compounds: pentabromophenol (PBP), triiodophenol (TIP), pentachlorophenol (PCP), tetrabromobisphenol A (Br4-BPA), tetrachlorobisphenol A (Cl4-BPA), ioxynil, tribromophenol (TBP) and trichlorophenol (TCP). To estimate the relative potency of the test chemicals against T3, the competition curve for unlabeled T3 (broken line) was inserted. Non-specific binding, which was less than 6% of total binding, was subtracted from total binding to give values for specific binding. Each value is the mean of triplicate determinations. These experiments were repeated at least three times.

Fig. 7. Scatchard plots of [125 I]T3 binding to recombinant skate transthyretin (TTR). For high-affinity and low affinity sites, TTR was incubated with 0.1 nM [125 I]T3 M in the presence or absence of various concentrations of unlabeled excess T3 (5 μ M) in the incubation buffer with (●; 5.3 nM TTR) or without (○; 0.53 nM TTR) 1 mM EDTA for 1 h at 4°C. For high-affinity sites, TTR (0.53 nM) was incubated with [125 I]T3 ranging from 0.05 to 0.4 nM in the presence or absence of unlabeled excess T3 (5 μ M) in the incubation buffer for 1 h at 4°C (*inset*). Non-specific binding, which was 12–16% of total binding, was subtracted from total binding to give values for specific binding. Each value is the mean of triplicate determinations. These experiments were repeated three or four times. v, molar ratio of bound [125 I]T3 to TTR concentration.

Fig. 8. Time course of enzyme-dependent 5-hydroxyisourate (HIU) hydrolysis by recombinant rainbow trout 5-hydroxyisourate hydrolase (HIUHase) and recombinant skate transthyretin (TTR). HIUHase, TTR and blank (buffer) were added to the reaction solution containing HIU (*arrow*). The hydrolysis of HIU was monitored spectrophotometrically at 312 nm as described in the Materials and Methods. Enzyme-dependent HIU hydrolysis indicates the difference in absorbance at 312 nm between the samples in the absence (spontaneous degradation of HIU) and the presence of HIUHase or TTR (spontaneous + enzymatic degradation of HIU). These experiments were repeated at least three times, with similar results.

Table 1. Relative potency of ligand binding to skate transthyretin.

Compounds	IC ₅₀	±	SEM	(n)		Relative potency [*]
	(nM)					
3,3',5-Triiodo-L-thyronine	2.0	±	0.1	(3)	a,b**	1
L-Thyroxine	26	±	5	(3)	d	0.078
3,3',5'-Triiodo-L-thyronine	81	±	6	(3)	e	0.025
3,3',5-Triiodo-D-thyronine	4.4	±	0.3	(3)	b,c	0.46
3,3',5-Triiodothyroacetic acid	1.0	±	0.2	(3)	a	2.0
3,3',5,5'-Tetraiodothyroacetic acid	9.7	±	0.8	(3)	c	0.21
3,5-Diiodo-L-thyronine	250	±	60	(3)	f	0.008
3,5-Diiodo-L-tyrosine	1000	>>		(3)		
3-Doiodo-L-tyrosine	1000	>>		(3)		
Tetrachlorobisphenol A	27	±	5	(4)	d,e,f,g	0.074
Tetrabromobisphenol A	9.8	±	2.3	(4)	c,d,e	0.20
Pentachlorophenol	5.1	±	0.5	(3)	b,c	0.39
Pebtabromophenol	1.4	±	0.3	(4)	a	1.4
Triiodophenol	2.2	±	0.4	(3)	a,b	0.91
Tribromphenol	49	±	11	(3)	g	0.04
Trichlorophenol	44	±	8	(3)	f,g	0.05
Ioxynil	33	±	6	(3)	e,f,g	0.06

*Relative potency is given as the ratio of IC₅₀ for T3 to IC₅₀ for a chemical.

**Different letters indicate significantly different means and were determined by one-way analysis of variance with Scheffe's test for multiple comparisons ($p < 0.01$).

Table 2. Kinetic parameters of T3 binding to the recombinant little skate transthyretin.

Chelator	Class	K_d (nM)	MBC (pmol/ μ g protein)
(-) EDTA	two classes (high affinity site)	0.52 ± 0.03 (4)	2.2 ± 1.0 (4)
	(low affinity site)	17 ± 3 (4)	19 ± 5 (4)
	one class (high affinity site)	0.24 ± 0.04 (4)	3.7 ± 1.1 (4)
(+) EDTA	one class	21 ± 1 (3)	1.4 ± 0.1 (3)

Each value is the mean $\pm$ standard error of the mean (n).

Table 3. **Number of** divalent cations in recombinant little skate transthyretin.

	Methods of preparations	
	HIC column	Co-resin
Zn	4.47	6.25
Cu	2.29	0.36
Ni	<0.06	0.40
Co	<0.06	(0.48)
Fe	nd	<0.02
Total metal	6.76	7.01

TTR was prepared by hydrophobic interaction column (HIC) or Co-affinity resin chromatography and then gel filtration chromatography. Data are shown as metal/TTR (mol/mol). Each value is the mean ($n = 3$), with less than 1% variation.

*When Co-affinity chromatography was used, it is likely that the TTR samples were contaminated with Co^{2+} released from the Co-affinity resin. Therefore, these values are not included in the total metal contents.

nd, not determined.

Fig. 1-8 rev(color)

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

A

B

Fig. 7

Fig. 8

Figs 5-6 (BW)
Fig. 5

Fig. 6

A

B