Characterization of little skate (Leucoraja erinacea) recombinant transthyretin: Zinc-dependent 3,3′,5-triiodo-l-thyronine binding

1   Characterization of little skate (*Leucoraja erinacea*) recombinant transthyretin:

2   zinc-dependent 3,3',5-triiodo-L-thyronine binding

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4   Shunsuke Suzuki [a†], Kentaro Kasai [a†], Kiyoshi Yamauchi [a,b*]

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6   [a]Department of Biological Science, Graduate School of Science, Shizuoka University, Shizuoka 422-8529,

7   Japan

8   [b]Green Biology Research Division, Research Institute of Green Science and Technology, Shizuoka

9   University, Shizuoka 422-8529, Japan

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11

12   †Equally contributing author.

13   *Corresponding author.   Green Biology Research Division, Research Institute of Green Science and

14   Technology, Shizuoka University, Shizuoka 422-8529, Japan. Tel.: +81 54 238 4777, fax: +81 54 238

15   0986.

16   *E-mail address*: fgyfm536@gmail.com (S. Suzuki), mitsuya59451192@gmail.com (K. Kasai),

17   sbkyama@ipc.shizuoka.ac.jp (K. Yamauchi).

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19   *Abbreviations*: BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; HIU,

20   5-hydroxyisourate; HIUHase, 5-hydroxyisourate hydrolase; HPLC, high-performance liquid

21   chromatography; IC_{50}, 50% inhibitory concentration; ICP-OES, inductively coupled plasma optical

22   emission spectroscopy; IPTG, isopropyl β-D-1-thiogalactopyranoside; K_d, dissociation constant; MBC,

23   maximun binding capacities; PAGE, polyacrylamide-gel electrophoresis; reverse T3 or rT3,

24   3,3',5'-triiodo-L-thyronine; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; T3,

25   3,3',5-triiodo-L-thyronine; T4, L-thyroxine; TH, thyroid hormone; TTR, transthyretin.

26

27

28 **Abstract**

29 Transthyretin (TTR) diverged from an ancestral 5-hydroxyisourate hydrolase (HIUHase) by gene

30 duplication at some early stage of chordate evolution. To clarify how TTR had participated in the thyroid

31 system as an extracellular thyroid hormone (TH) binding protein, TH binding properties of recombinant

32 little skate *Leucoraja erinacea* TTR was investigated. At the amino acid level, skate TTR showed 37–46%

33 identities with the other vertebrate TTRs. Because the skate TTR had a unique histidine-rich segment in the

34 N-terminal region, it could be purified by Ni-affinity chromatography. The skate TTR was a 46-kDa

35 homotetramer of 14.5 kDa subunits, and had one order of magnitude higher affinity for

36 3,3',5-triiodo-L-thyronine (T3) and some halogenated phenols than for L-thyroxine. However, the skate

37 TTR had no HIUHase activity. Ethylenediaminetetraacetic acid (EDTA) treatment inhibited $[^{125}I]$T3

38 binding activity whereas the addition of Zn^{2+} to the EDTA-treated TTR recovered $[^{125}I]$T3 binding activity

39 in a Zn^{2+} concentration-dependent manner. Scatchard analysis revealed the presence of two classes of

40 binding site for T3, with dissociation constants of 0.24 and 17 nM. However, the high-affinity sites were

41 completely abolished with 1 mM EDTA, whereas the remaining low-affinity sites decreased binding

42 capacity. The number of zinc per TTR was quantified to be 4.5–6.3. Our results suggest that skate TTR has

43 tight Zn^{2+}-binding sites, which are essential for T3 binding to at least the high-affinity sites. Zn^{2+} binding to

44 the N-terminal histidine-rich segment may play an important role in acquisition or reinforcement of TH

45 binding ability during early evolution of TTR.

46

47 *Keywords*: transthyretin, thyroid hormone, divalent cation, endocrine disruption, 5-hydroxyisourate

48 hydrolase, *Leucoraja erinacea*

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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

81   fishes, we found TTR cDNA in the little skate *Leucoraja erinacea* database (http://skatebase.org), and

82   noticed that such unidirectional changes may not be true for the skate TTR. Because the skate TTR has a

83   highly hydrophilic stretch containing four histidine residues in the N-terminal region as if the skate TTR

84   has a histidine tag. From this unique structure we supposed that functional analyses of the TTR gene

85   superfamily in primitive gnathostomes may provide a good model for understanding the processes of

86   neo-functionalization found in duplicated genes.

87   As a first step towards elucidating how TTR has participated in the thyroid system as a TH-binding

88   protein during vertebrate evolution, we investigated TH binding properties and HIUHase activity of the

89   recombinant skate TTR.

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91

92   **2. Materials and Methods**

93   *2.1. Reagents*

94   3,3'[^{125}I],5-Triiodo-L-thyronine (81 TBq/mmol, carrier free) was purchased from PerkinElmer

95   (Waltham, MA, USA). 3,3',5-Triiodo-L-thyronine, T4, D-T3, 3,3',5'-triiodo-L-thyronine (reverse T3 or

96   rT3), 3,3',5-triiodothyroacetic acid, 3,3',5,5'-tetraiodothyroacetic acid, diiodo-L-tyrosine,

97   monoiodo-L-tyrosine, benzo[*a*]pyrene and pentachlorophenol were obtained from Sigma-Aldrich (St. Louis,

98   MO, USA). Bisphenol A, 2,4,6-triiodophenol, 2,4,6-tribromophenol, 2,4,6-trichlorophenol,

99   pentabromophenol, *o-t*-butylphenol, mirex, malathion (diethyl

100   2-dimethoxyphosphinothioylsulfanylbutanedioate, analytical standard), benzophenone, *n*-butylbenzyl

101   phthalate, bis(2-ethylhexyl) adipate, bis(2-ethylhexyl) phthalate, dicyclohexyl phthalate, di-*n*-butyl

102   phthalate, tributyltin (IV) chloride, α-benzoepin (α-endosulfan;

103   6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9,methane-2,4,3-benzodiathiepine), dicofol (Kelthane,

104   2,2,2-trichloro-1,1-bis(4-chloro-phenyl)ethanol, analytical standard), urea, *Candida* sp. uricase and

105   trimethylamine N-oxide were purchased from Wako Pure Chemical Industries (Osaka, Japan).

106   3,3',5,5'-Tetrabromobisphenol A, 3,3',5,5'-tetrachlorobisphenol A and 3,5-diiodo-L-thyronine were from

107   Tokyo Chemical Industry (Tokyo, Japan). 2,4-Dichlorophenoxyacetic acid, 4-nonylphenol, di-2-ethyl

108   phthalate and 2,4-dinitrophenol were purchased from Kanto Chemicals (Tokyo, Japan). Isopropyl

109 β-D-1-thiogalactopyranoside (IPTG) was obtained from Nacalai Tesque (Kyoto, Japan). 2-Isopropylphenol

110 was from Lancaster. Methoprene (isopropyl (E,E')-(RS)-11-methoxy-3,7,11-trimethyldodeca-2,4-dienoate)

111 was from Ehrenstorfer Quality (Augsburg, Germany). Ioxynil (3,5-diiodo-4-hydroxybezonitril, analytical

112 standard) and acetochlor (2-chloro-N-(ethoxymethyl)-N-(2-ethyl-6-methylphenyl)acetamide) were obtained

113 from Riedel-de Haën (Seeize, Germany). Ni-resins were from Bio-Rad (Ni-IMAC Profinity, Hercules, CA,

114 USA) and Invitrogen (ProBond Nickel-Chelating Resin, Carlsbad, CA, USA). Co-resin was obtained from

115 Clontech (Talon Metal Affinity Resin, Mountain View, CA, USA). All other chemicals used in this study

116 were either chromatography grade or the highest grade available and were purchased from Wako Pure

117 Chemical Industries or Nacalai Tesque.

118   All chemicals tested as endocrine disrupting chemicals were dissolved in dimethylsulfoxide or 0.5 N

119 NaOH to concentrations of 2–5 mM. These chemicals were then diluted with an appropriate buffer. For

120 TTR pull-down assay, a blank assay without proteins with solvent alone at less than 1.0% (v/v) was done.

121 The solvent did not affect the recovery of TTR in the pull-down assay. For competitive [125I]T3 binding

122 assay, a control assay without test chemicals was performed in the presence of the solvent alone and at less

123 than 0.4% (v/v). The solvent did not affect the competitive [125I]T3 binding assay.

124

125 *2.2. Protein expression and purification*

126   The skate TTR cDNA (accession no. CV221819, 607 bp) was amplified by polymerase chain reaction

127 (PCR) with sense primer 5'-GTT<u>CATATG</u>CCACATAGTCACGGCGACC-3' (64–83) with a recognition

128 site for *Nde*I (underlined), and antisense primer 5'-GTA<u>GGATCC</u>GGTTTTCACGAGGTGTTAAG-3'

129 (468–449) with a recognition site for *Bam*HI (underlined). PCR was carried out in 50 μL of solution

130 containing 0.2 mM dNTP, 0.2 μM of each primer, 1 ng template DNA and 1.25 U DNA polymerase

131 (PrimeSTAR HS, TAKARA, Siga, Japan), using the following protocol: 98°C (15 s), and 20 cycles of

132 98°C (10 s), 65°C (5 s) and 72°C (30 s), followed by 72°C (5 min). Amplicons (64–449) were cloned

133 between the *Nde*I and *Bam*HI sites of a pET3a expression vector (Novagen, Madison, WI). Plasmids for

134 expression were transformed into *Escherichia coli* Rosetta 2(DE3)pLysS (Novagen). Bacteria were usually

135 grown in 50 mL LB medium with 50 μg/mL ampicillin and 34 μg/mL chloramphenicol at 37°C until the

136 absorbance at 600 nm reached 0.8. The temperature was lowered to 18°C, 0.1 mM IPTG was added, and

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

138 until used.

139   After suspending the bacterial pellet with 5 mL of homogenization buffer (1 mM imidazole, 0.3 M

140 NaCl, 50 mM sodium phosphate, pH 8.0, 1 mM benzamidine hydrochloride, and 1 mM

141 phenylmethylsulfonyl fluoride), the cells were disrupted by sonication 20 times for 10 s on ice at the range

142 of 5 (UltraS homogenizer, VP-30S, TAITEC, Saitama, Japan), and the lysate was centrifuged at $18,000 \times g$

143 for 15 min at 4°C. Recombinant proteins were isolated from the other proteins in the supernatant by nickel

144 or cobalt affinity chromatography (0.5 mL of the resin), by stepwise elution with various concentrations of

145 imidazole (5, 20, 60, 150, 250 and 500 mM) in 0.3 M NaCl and 50 mM sodium phosphate, pH 8.0. Peak

146 fractions were immediately applied to a Cellulofine GCL-2000 sf column (1.5×93 cm, Seikagaku Co.,

147 Tokyo, Japan), which had been equilibrated with 20 mM Tris, pH 7.5. Purified protein was stored in 10%

148 glycerol at -85°C for later use. The yields of the recombinant proteins were 0.6–0.8 mg/50 mL bacterial

149 culture. The recombinant rainbow trout HIUHase with three histidine residues at the N-terminal end and

150 the recombinant skate TTR were also purified by ammonium sulfate precipitation, hydrophobic interaction

151 chromatography on Phenyl Cellulofine column (1.0×10 cm, Seikagaku Co.) and then gel filtration

152 chromatography on Cellulofine GCL-2000 sf column, as described previously (Kasai et al., 2013).

153

154 *2.3. Protein analyses*

155   Protein concentration was measured by the micro-Lowry method (Jain et al., 2002) with bovine serum

156 albumin (BSA) as the standard. Proteins was electrophoresed on an sodium dodecyl sulfate

157 (SDS)-polyacrylamide gel (15%) (Laemmli, 1970) with molecular markers: phosphorylase b (97 kDa),

158 BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and

159 α-lactalbumin (14.4 kDa). The gel was stained and proteins were visualized with Coomassie Brilliant Blue.

160   The molecular mass of TTR was estimated by high-performance liquid chromatography (HPLC) on

161 YMC-Pack Diol 120 (500×8.0 mm, YMC, Kyoto, Japan) and YMC-Guardpack Diol-12 (30×8.0 mm),

162 which were pre-equilibrated and eluted with 50 mM Tris-HCl, pH 7.5, and 140 mM NaCl, at flow rate of 1

163 mL/min at 25°C.

164

165 *2.4. Ligand binding assay using a metal chelate affinity resin (TTR pull-down assay)*

166   Iodothyronine or chemical binding was carried out by the TTR pull-down assay using a metal chelate

167 affinity resin (Kasai et al., 2013). Briefly, TTR (0.5 μM) was incubated with iodothyronines or chemicals

168 (0.5 μM) in 500 μL of Tris-buffered saline, pH 7.5, for 1 h at 4°C, and the mixture was further incubated

169 with Co-resin (5 μL bed volume) for 1 h at 4°C in a 600-μL plastic tube using a rotator. After a brief

170 centrifugation, the TTR-ligand complex bound to the resin in the pellet was immediately recovered by the

171 addition of 20 μL of elution buffer (500 mM imidazole, 0.3 M NaCl, and 50 mM sodium phosphate, pH

172 8.0). The TTR-ligand complex released from the resin was mixed with the same volume of 1% acetic acid

173 in methanol to extract the ligand from the complex. The mixture was agitated then centrifuged at

174 $12,000 \times g$ for 10 min at 4°C. The ligand (15 μL) in the supernatant was analyzed by reverse phase-HPLC

175 on a C_{18} analytical column (Mightysil RP-18 GP, 250 mm × 4.6 mm, 5 mm particle diameter, Kanto

176 Chemical, Tokyo, Japan) with a UV detector at 254 nm, as described previously (Kasai et al., 2013). The

177 amount of the ligand was quantified by comparison with the standards (4–100 pmol), with the lower limits

178 of quantitation for iodothyronines and environmental chemicals of 2.0–7.2 pmol and 6–14.2 pmol,

179 respectively, which were three times higher than the lower limits of detection.

180

181 *2.5. [^{125}I]T3 binding assay*

182   Unlike bony fishes, cartilaginous fishes retain a high concentration of organic osmolytes, such as urea

183 (300–400 mM), in their body fluids to maintain a high plasma osmolality that is similar to that in the

184 external environment. To counteract destabilization of proteins by urea, cartilaginous fishes also have

185 methylamines, e.g., trimethylamine N-oxide (Yancey, 2005). Recombinant TTR (7.5 ng/tube, 0.53 nM)

186 was incubated with 0.1 nM [^{125}I]T3 in 250 μL of buffer (20 mM Tris, pH 7.5, 280 mM NaCl, 360 mM urea,

187 and 90 mM trimethylamine N-oxide) in the presence or absence of test compounds (cations, unlabeled

188 iodothyronines or environmental chemicals) for 1.0 h at 4°C, unless otherwise noted. In some cases,

189 various concentrations of TTR were pre-incubated with 1 mM ethylenediaminetetraacetic acid (EDTA).

190 After dilution with the buffer, TTR (0.53 nM) was used for [^{125}I]T3 binding assay in the presence or

191 absence of cations. For the Scatchard plot of T3 binding to both high-affinity and low-affinity sites, skate

192 TTR was incubated with 0.1 nM [^{125}I]T3 in the presence or absence of increasing concentrations of

193   unlabeled T3. The dissociation constant (K_d) value for high affinity sites was expected to be 10^{-10} M from

194   this experiment. To estimate more accurately the K_d value, TTR was incubated with [^{125}I]T3 (0.052 to 0.4

195   nM) in the presence or absence of 5 μM of unlabeled T3. [^{125}I]T3 bound to proteins was separated from

196   free [^{125}I]T3 by the polyethyleneglycol method (Yamauchi et al., 1993). The radioactivity of the samples

197   was measured in a gamma counter (2480 WIZARD^2; PerkinElmer). The amount of [^{125}I]T3 bound

198   non-specifically was derived from the radioactivity of samples incubated with 5 μM unlabeled T3. The

199   non-specific binding value was subtracted from the amount of total bound [^{125}I]T3 that was derived from

200   the radioactivity of samples incubated without unlabeled T3, to give the value of specifically bound

201   [^{125}I]T3. The K_ds and maximum binding capacities (MBCs) for T3 binding were determined from

202   Scatchard plots for a single class (Scatchard, 1949) or for two classes (Rosenthal, 1967).

203

204   *2.6. Determination of the average number of divalent cations per TTR*

205   TTR samples were prepared by Co-affinity chromatography and gel filtration chromatography on

206   Cellulofine GCL 2000-sf column. Alternatively, to avoid the effects of Co-resin on the metal contents of

207   TTR, TTR samples were also prepared by ammonium sulfate precipitation, hydrophobic interaction column

208   chromatography, and gel filtration column chromatography, as shown previously (Kasai et al., 2013). The

209   content of divalent cations in TTR samples (1.5 mL in 4 mM Tris, pH 7.5, 0.16–0.65 mg/mL) was

210   quantified three times by inductively coupled plasma optical emission spectroscopy (ICP-OES) (Optima

211   3300 DV, PerkinElmer) using calibration curves of zinc, copper, nickel, cobalt and iron standards (0, 0.01,

212   0.05, 0.1, 0.5, 1.0, 5.0 mg/L in 0.1 mol/L HNO_3) (Wako). The lower limits of quantitation were 0.011

213   mg/mL, and the lower limits of detection were 0.002 mg/mL.

214

215   *2.7. HIU hydrolysis assay*

216   HIU hydrolysis assay was conducted according to the previous method (Kasai et al., 2013). Enzyme

217   activity was measured at 25–27°C by following the decrease in absorbance at 312 nm as HIU was

218   hydrolyzed (Lee et al., 2005). 5-Hydroxyisourate was generated *in situ* by the addition of 50 μL of 0.32 μM

219   uricase in 50 mM potassium phosphate buffer, pH 7.5, to 400 μL of 100 μM uric acid in the same buffer.

220   When the amount of HIU reached a maximum (2.4 min after starting the reaction), 10 μL of HIUHase (2

221 μg) or TTR (2 μg) was added to the mixture in a microcuvette. Absorbance at 312 nm was immediately

222 monitored at 0.2 min intervals using a spectrophotometer (U-3210, Hitachi, Japan). The control assay was

223 carried out similarly except that HIUHase or TTR solution was replaced by phosphate buffer. As HIU is

224 not stable, changes in absorbance at 312 nm does not reflect correctly the enzymatic hydrolysis of HIU.

225 Therefore, the enzyme activity was expressed as the difference in absorbance at 312 nm between the

226 presence (spontaneous + enzymatic hydrolysis of HIU) and the absence (spontaneous hydrolysis of HIU) of

227 proteins.

228

229 *2.8. Statistics*

230 The data are the mean ± standard error of the mean ($n \geq 3$), unless otherwise noted. Differences

231 between groups were analyzed by a one-way analysis of variance, with the Scheffe's test for multiple

232 comparisons. $P < 0.05$ was considered statistically significant.

233

234

235 **3. Results**

236 *3.1. Comparison of TTR amino acid sequences*

237 The skate TTR amino acid sequence shared 37–46% identities with the other vertebrate TTR amino

238 acid sequences shown in Fig. 1, although the skate TTR amino acid sequences shared only 24–27%

239 identities with the amphioxus and trout HIUHase amino acid sequences (data not shown). Significant

240 variations of amino acid residues were detected at the N-terminal regions, where the skate and lamprey

241 TTR had a histidine-rich segment of high hydrophilicity, HSHGDHH and HKSHESH, respectively. Thirty

242 residues were invariant in the amino acid sequences among the 9 TTR sequences.

243

244 *3.2. Molecular features of recombinant skate TTR proteins*

245 Skate TTR could be purified by nickel-affinity chromatography (Fig. 2A) and gel filtration

246 chromatography. Skate TTR molecule may be a slightly compact tetramer. The molecular mass of the skate

247 TTR under native conditions was estimated to be ~46 kDa by gel filtration column chromatography (Fig.

248 2B). This value was somewhat smaller than the molecular mass of the tetramer (56.8 kDa) estimated from

249   the amino acid sequence. The molecular mass for the TTR polypeptide was 14.5 kDa on

250   SDS-polyacrylamide-gel electrophoresis (PAGE) (Fig. 2C).

251

252   *3.3. Binding of iodothyronines and environmental chemicals to recombinant skate TTR using TTR*

253   *pull-down assay*

254   3,3',5-Triiodo-L-thyronine bound to skate TTR then extracted with methanol was eluted at the same

255   retention time (11.46 min) on reverse phase-HPLC as the T3 standard, as shown previously (Kasai et al.,

256   2013). The peak of T3 was negligible when the same pull-down assay was done without TTR (blank).

257   Skate TTR exhibited high binding activity for natural ligands T3 and T4, and related compounds, with the

258   following rank order affinity: D-T3 ≥ 3,3',5,5'-tetraiodothyroacetic acid, T3, T4 and

259   3,3',5-triiodothyroacetic acid ≥ reverse T3 > 3,5-diiodo-L-thyronine (Fig. 3A). With regard to

260   environmental chemicals (Fig. 3B), skate TTR showed binding activity for triiodophenol and ioxynil, but

261   not for benzophenone and 2,4-dinitrophenol.

262

263   *3.4. [^{125}I]T3 binding activity of recombinant skate TTRs*

264   *3.4.1. Effects of divalent cations*

265   To test the possibility that the skate TTR has significant affinity for metal ions, [^{125}I]T3 binding was

266   performed in the presence of 1 mM divalent cations. However, any cations tested did not show statistically

267   significant effects on [^{125}I]T3 binding (Fig. 4A). We next pre-incubated skate TTR (0.53–2640 nM) with

268   EDTA (1 mM) at various molar ratios followed by [^{125}I]T3 binding assay at 0.53 nM of TTR, to determine

269   the most effective molar ratio of TTR/EDTA for eliciting the chelating action of EDTA (Fig. 4B). [^{125}I]T3

270   binding was decreased by more than a half at the TTR pre-incubation concentration of 264 nM

271   (TTR/EDTA molar ratio of 1/3,800), and by about 90% at the TTR pre-incubation concentration of 26.4

272   nM (TTR/EDTA molar ratio of 1/38,000). Using the TTR pre-treated with 1 mM EDTA at the TTR/EDTA

273   molar ratio of 1/38,000, the effects of divalent cations (1 mM) on [^{125}I]T3 binding to TTR (0.53 nM) was

274   investigated (Fig. 4C). Zn^{2+}, Ni^{2+} and Mn^{2+} (each 1 mM) recovered [^{125}I]T3 binding activity up to

275   60–90% of that of the TTR not pre-treated with EDTA. Ca^{2+} and Mg^{2+} had little effect or were not effective.

276   The effects of Fe^{2+}, Co^{2+}, and Cu^{2+} on [^{125}I]T3 binding could not be determined, because of the formation

277 of insoluble materials under our assay conditions (data not shown). Addition of 20 μM Zn^{2+} activated

278 partially [^{125}I]T3 binding in spite of the presence of the residual EDTA (final 20 μM; Fig 4D). [^{125}I]T3

279 binding activity reached a plateau at more than 50 μM Zn^{2+}.

280

281 *3.4.2. Competitive inhibition of [^{125}I]T$_3$ binding to recombinant skate TTR by iodothyronines and other*

282 *compounds*

283   [^{125}I]T$_3$ binding was investigated in the presence of various concentrations of iodothyronines and their

284 related compounds (Fig. 5). The most powerful competitor was 3,3',5-triiodothyroacetic acid with 50%

285 inhibitory concentration (IC_{50}) of 1.0 ± 0.2 nM, which was two times less than the IC_{50} for T3 (Table 1).

286 The rank order binding affinity was 3,3',5-triiodothyroacetic acid $\geq$ T3 $\geq$

287 D-T3 $\geq$ 3,3',5,5'-tetraiodothyroacetic acid > T4 > reverse T3 > 3,5-diiodo-L-thyronine > diiodo-L-tyrosine

288 and monoiodo-L-tyrosine. T3 was 13 times more potent than T4. Acetic acid analogs

289 (3,3',5-triiodothyroacetic acid and 3,3',5,5'-tetraiodothyroacetic acid) were 2-3 times more potent than the

290 corresponding iodothyronines (T3 and T4). Iodotyrosines had no affinity for skate TTR.

291   As a primary screening of environmental chemicals that interfere with [^{125}I]T$_3$ binding to skate TTR in

292 vitro, inhibitory effects of chemicals on [^{125}I]T$_3$ binding was investigated at 1 μM concentration. Out of 29

293 chemicals tested, 8 chemicals, all of which were phenolic compounds consisting of one or two phenolic

294 rings with several halogen atoms, strongly inhibited [^{125}I]T$_3$ binding (Fig. 6A). Phenolic compounds

295 without halogen atoms, phthalates and the other chemicals tested were not significant competitors, even if

296 they had benzene or phenolic ring(s).

297   [^{125}I]T$_3$ binding in the presence of varying concentrations of the eight chemicals that strongly inhibited

298 [^{125}I]T$_3$ binding was tested (Fig. 6B). The most effective inhibitor of [^{125}I]T$_3$ binding to skate TTR was

299 pentabromophenol, with an IC_{50} of 1.4 ± 0.3 nM, which was between those for 3,3',5-triiodothyroacetic

300 acid and T3 (Table 1). The rank order binding affinity was pentabromophenol $\geq$ triiodophenol $\geq$

301 pentachlorophenol $\geq$ 3,3',5,5'-tetrabromobisphenol A $\geq$ 3,3',5,5'-tetrachlorobisphenol A and ioxynil $\geq$

302 trichlorophenol and tribromophenol.

303

304 *3.4.3. Kinetics of [^{125}I]T$_3$ binding to skate TTR*

305   Scatchard plots revealed the presence of two classes of binding sites for T3 (Fig. 7), with K_{d1} and K_{d2}

306   of 0.52 ± 0.03 nM and 17 ± 3 nM, and MBC$_1$ and MBC$_2$ of 2.2 ± 1.0 pmol/μg protein and 19 ± 5 pmol/μg

307   protein, respectively. To determine more precisely the K_{d1} value, we conducted Scatchard analysis at lower

308   concentrations and within a more narrow range of [^{125}I]T3, which showed the K_{d1} value of 0.24 ± 0.04 nM

309   (Fig. 7 *inset*). In the presence of 1 mM EDTA, the Scatchard plot showed a single class of binding sites,

310   where high-affinity sites were completely abolished. The K_d value was estimated to be 21 ± 1 nM, which

311   nearly corresponded to that for the low-affinity sites of the TTR untreated with EDTA, with decreased

312   MBC value, 1.4 ± 0.1 pmol/μg protein (Table 2).

313

314   *3.5. HIU hydrolysis activity of recombinant skate TTR and recombinant rainbow trout HIUHase*

315   As the production of HIU, a substrate for HIUHase, reached a peak at 2.4 min after the addition of

316   uricase into the reaction mixture (Fig. 8), we investigated HIUHase activity from this time point by adding

317   trout HIUHase or skate TTR. Hydrolysis of HIU by the trout HIUHase was clearly detected at the

318   concentration of 4.4 μg/mL. Activity (difference in absorbance at 312 nm between the samples in the

319   absence and the presence of HIUHase) peaked at 2.4–4.0 min and decreased thereafter. However, there was

320   no significant effect of the skate TTR on the hydrolysis of HIU at the same concentration (Fig. 8).

321

322   *3.6*. Contents of divalent cations in skate TTR molecule

323   The zinc content measured by using ICP-OES was 4.47–6.25 mol/mol TTR (Table 3). Additionally,

324   nickel and copper were also detected at 0.40 and 0.36–2.29 mol/mol TTR, respectively. The contents of

325   cobalt and iron were below detectable levels. The TTRs purified using Co-resin and hydrophobic

326   interaction chromatography had similar total metal contents, 6.76–7.01 mol/mol TTR. The same fractions

327   on gel chromatography of the cell extract, which were obtained from *E. coli* harboring intact pET3a, did

328   not contain detectable levels of the metals analyzed.

329

330

331   **4. Discussion**

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

333 supported by the presence of a histidine-rich segment in the N-terminal region, the ability to be purified by

334 metal chelate affinity chromatography, inactivation of T3 binding by EDTA and recovery of T3 binding

335 activity by divalent cations. Skate TTR had high-affinity and low-affinity sites for T3 with K_ds of 0.24 nM

336 and 17 nM, but not HIU hydrolysis activity. All of the high affinity and most of the low affinity sites for T3

337 were abolished by the treatment of EDTA. Analysis by IPC-OES revealed that the skate TTR molecule

338 contains 4.47–6.25 zinc ions. Therefore, zinc ion may be essential for the skate TTR to bind T3.

339   We discovered that a histidine-rich segment, HSHGDHH, located in the N-terminal region of skate

340 TTR may act as a natural histidine tag, which facilitated the purification of this protein by Ni-affinity

341 chromatography and may also provide a novel ligand binding assay (TTR pull-down assay) for

342 investigating the ligand interaction. The histidine-rich segments of the skate and lamprey TTRs resemble

343 the zinc-binding motif of metalloproteases (HEXXH or HEXXHXXGXXH/D) (Cerdà-Costa and

344 Gomis-Rüth 2014), but are quite different from the metal-binding motif of metallothionein with

345 cysteine-rich motifs (Vašák aand Meloni, 2011). TTR is a homotetramer consisting of a dimer of dimers

346 (subunits A and B, and subunits C and D). As the N-terminal regions of two subunits (A and D, or B and C)

347 are located in the vicinity of the entrance of either hormone binding pocket (Hamilton et al., 1993), the two

348 histidine-rich segments in the N-terminal regions are highly likely to act as a histidine tag comparable with

349 the ordinary 6× histidine-tag. In this study, we do not exclude the possibility of the presence of several

350 binding sites for divalent cations in skate TTR molecule besides the N-terminal region.

351   Divalent cation binding to skate TTR is necessary to elicit strong T3 binding activity that may occur at

352 physiological conditions, in which Zn^{2+} is the best possible candidate cation. [125I]T3 binding to skate TTR

353 was inactivated by EDTA and re-activated by divalent cations in vitro. When skate TTR (264 nM) was

354 pre-treated with EDTA (1 mM) at the molar ratio of 1/3,800, [125I]T3 binding to the skate TTR was

355 partially inhibited. This suggests that most of the zinc ions involved in T3 binding do not dissociate from

356 the TTR of the micromolar range. The concentration of Zn^{2+} that was needed to reactivate the [125I]T3

357 binding activity, ~0.05 mM, was less than the physiological range (0.1–0.2 mM) of Zn^{2+} in fish plasma

358 (Sturrock et al. 2013). Scatchard analysis revealed the presence of two classes of binding sites. [125I]T3

359 binding to all of the high affinity sites and most of the low affinity sites were lost with the addition of 1

360 mM EDTA. This finding leads us to the possibilities that Zn^{2+} binding is essential for skate TTR to express

361   at least high affinity sites for T3 binding and that Zn^{2+} at the concentration in plasma may modulate T3

362   binding to skate TTR in vivo. In this study we determined the average number of metal ions per TTR. If

363   skate TTR samples consist of a mixture of the TTR components with different content of Zn^{2+} or the Zn^{2+}

364   content of skate TTR in circulation differs locally, a variety of the number of Zn^{2+} per TTR may increase

365   the functional diversity in T3 binding activity. Future study will need to address the physiological meanings

366   of Zn^{2+} binding to TTR on the skate thyroid system.

367   This is the first report showing the divalent-dependent TH binding activity of TTR, although there are

368   several reports regarding the effects of metal ions on the other functions of human TTR. Zn^{2+} binding

369   decreases monomer stability to enhance TTR aggregation at an acidic pH (Palmieri et al., 2010) or amyloid

370   formation of a mutant TTR (Wilkinson-White and Easterbrook-Smith, 2007), and decreases the affinity of

371   TTR for RBP4 (Palmieri et al., 2010). Zn^{2+} is also essential for expressing the metalloprotease activity of

372   human TTR, which was inhibited by EDTA or 1,10-phenanthroline and reactivated by divalent cations (Liz

373   et al., 2012). Zinc content in human TTR was ~0.2 per TTR molecule (Liz et al., 2012), which was one

374   order less than that in the skate TTR (4.47–6.25 per TTR). In addition, the skate TTR contained copper at

375   0.36–2.29 per TTR, and nickel at 0.4 per TTR. This means that four fifths of human TTR is independent of

376   zinc-dependent functions. However, in the skate TTR, because of the high content of zinc, T3 binding

377   activity which is highly zinc-dependent may be fully affected by the zinc content. Therefore, the deficiency

378   of metal ions such as zinc may influence the functions of TTR to greater extent in skates compared with

379   humans.

380   Zn^{2+} binding may be an ancient property of the TTR/HIUHase superfamily with high diversity. In the

381   TTR family, Zn^{2+} binding is experimentally shown so far in the skate and human protein alone. Among the

382   four Zn^{2+} binding sites (ZBS1 to ZBS4) proposed in human TTR (Palmieri et al., 2010), only ZBS1 (C10

383   and H56 in Fig. 1) is conserved in the vertebrate TTRs investigated. Among ten amino acid residues that

384   constitute the four Zn^{2+} binding sites in the human TTR, four are identical and one is similar to the

385   corresponding amino acid residues in the skate TTR. The histidine-rich segments found in the skate and

386   lamprey TTRs are not detected in the bony fish and tetrapod TTRs (Fig.1) or HIUHases of various

387   vertebrates. However, in the HIUHase family, our previous study (Kasai et al., 2013) showed that Zn^{2+} (50

388   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 Zn^{2+} 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 [^{125}I]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

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

418   Skate TTR has significant high affinity for several halogenated phenolic compounds that structurally

419 resemble THs (Howdeshell, 2002). Tetrabromobisphenol A and pentabromophenol were the most potent

420 chemicals in halogenated phenolic compounds with two phenolic rings and with a single ring, respectively.

421 The structural preference of ligand binding was well conserved in vertebrate evolution (Kudo et al., 2006;

422 Meerts et al., 2000; Morgado et al., 2007; Yamauchi et al., 2003). Furthermore, in the structure-activity

423 relationships, halogen species-dependency ($Br^- > Cl^-$ in pentahalogenated phenols; $I^- > Br^- \approx Cl^-$ in

424 trihalogented phenols; $Br^- > Cl^- >$ no halogen in bisphenol A) and halogen number-dependency (penta-

425 >tri- in brominated and chlorinated phenols) are similar with previous studies using human (Meerts et al.,

426 2000), chicken and amphibian TTRs (Kudo et al., 2006; Yamauchi et al., 2003).

427   Production of brominated phenols, used as a flame retardant or its intermediate, or as a wood

428 preservative, may result in the release of brominated phenols to the aquatic environment for several

429 decades (WHO, 2005). The annual consumption by humans of tetrabromobisphenol A is 89,400 tons in

430 Asia, which is 56% of the total market demand in 2001 (Birnbaum and Staskal, 2004). As skates spend

431 time in the sediments of the sea, where they feed on various invertebrates including decapod crustaceans,

432 amphipods and polychaete worms, they are ecologically situated in the top or middle of the benthic food

433 web. This ecological position may expose skates to hydrophobic and persistent organic pollutants retained

434 in the benthic environments with a relatively high bioaccumulation. Therefore, investigating the inhibitory

435 effects of persistent halogenated compounds on TH binding to skate TTR may be useful to assess the threat

436 on the thyroid system from environmental chemicals in benthic environments.

437   In conclusion, we have demonstrated that skate TTR is a strong T3-binder in vitro, and that T3

438 binding to skate TTR is divalent cation-dependent and competitively inhibited by environmental phenolic

439 compounds with halogens. The unique structure of a histidine-rich segment in the N-terminal region may

440 act as a natural histidine tag, raising the possibility that the TH binding activity of the skate TTR may be

441 modulated by Zn^{2+} in plasma. We proposed that a histidine-rich segment in the N-terminal region of TTR

442 may play an important role in acquisition or reinforcement of TH binding activity via metal binding during

443 early vertebrate evolution.

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457   **References**

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585 **Figure legends**

586 Fig. 1. Comparison of transthyretin (TTR) sequences with possible zinc-binding sites. Mature TTR amino

587 acid sequences from lamprey (Manzon et al., 2007), skate (accession no. CV221819), trout (Yamauchi et

588 al., 1999; accession.no.CX256523), seabream (Santos and Power, 1999), toad (Prapunpoj et al., 2000),

589 bullfrog (Yamauchi et al., 1993, 1998), crocodile (Prapunpoj et al., 2002), chicken (Duan et al., 1991) and

590 human (Kanda et al., 1974) were aligned. The N-terminal ends of the lamprey, skate and seabream TTRs

591 are predicted, whereas those of the other TTRs are based on Edman degradation. The numbering of amino

592 acid is based on that of human TTR. Features of secondary structure of human TTR (Hamilton et al., 1993)

593 are indicated above the lamprey sequence. Histidine-rich segments in the disordered N-terminal region of

594 the skate and lamprey TTRs are boxed. A segment that resembles a zinc-binidng motif of metalloprotease

595 (HEXXHXXGCCH/D) (Cerdà-Costa and Gomis-Rüth, 2014) is underlined. Four zinc-binding sites (ZBSs

596 1-4) proposed in human TTR (Palmieri et al., 2010) are also boxed: C10 and H56 for ZBS1, H88, H90 and

597 E92 for ZBS2, H31, D74 and E72 (or E62 at acidic pH), H31, E61 and D74 for ZBS4. Marks (*)

598 underneath the sequence alignment indicate positions where amino acid residues are invariant, among the 9

599 sequences. The numbers of histidine residues in the TTR sequences are shown on the right.

600

601 Fig. 2. Purification of recombinant skate transthyretin (TTR) by metal chelate affinity chromatography and

602 gel chromatography. (*A*) SDS-PAGE of the fractions of metal chelate affinity chromatography. The

603 recombinant skate TTR was separated from the other bacterial proteins in cell extract by metal chelate

604 affinity chromatography on Ni-resin, and then eluted with the buffer containing 150 mM imidazole (*an*

605 *arrow* in fraction *E4*). Lanes from left to right: *ppt*, pellet of cell lysate; *sup*, supernatant of cell lysate; *FT*,

606 flow-through fraction; *W1* to *W4*, washing fractions; *E1* to *E6*, elution fractions with buffer containing 5, 20,

607 60, 150, 250, 500 mM imidazole, respectively. (*B*) High performance gel chromatography of purified

608 recombinant skate TTR. Protein was applied to YMC Diol 120 High performance gel chromatography

609 column (8×500 mm) in 50 mM Tris-HCl, pH 7.5, and 140 mM NaCl, at 1 mL/min. Elution was monitored

610 at absorbance at 280 nm. Markers: Blue dextran (void volume, V_0); apoferritin (Fer, 440 kDa); alcohol

611 dehydrogenase (ADH, 158 kDa); bovine serum albumin (BSA, 68 kDa); ovalbumin (OVA, 45 kDa); and

612 myoglobin (Mb, 17 kDa). (*C*) SDS-PAGE of the peak fraction of Cellulofine GCL-2000 sf gel filtration

613 chromatography. *Arrow*, band of skate TTR.

614

615 Fig. 3. Ligand-binding to recombinant skate transthyretin (TTR) determined by the TTR pull-down assay

616 using metal chelate affinity resin. After incubating TTR (14.2 μg, 0.5 μM) with each ligand (0.5 μM) (*A*,

617 iodothyronines and their acetic acid analogs; *B*, environmental chemicals) in 500 μL of the incubation

618 buffer, the ligand bound to TTR was analyzed by reverse phase-HPLC as described in the Materials and

619 methods. The vertical axis of graphs indicates the amount of the bound ligand that was recovered in 40 μL

620 of the extraction buffer. Blank data obtained from samples in the absence of TTR were subtracted from

621 those obtained from samples in the presence of TTR. Each value is the mean ± standard error of the mean

622 (*n* = 5–6 for *panel A*; *n* = 3 for *panel B*). Different letters indicate significantly different means (*p* < 0.01). T3,

623 3,3',5-triiodo-L-thyronine; T4, L-thyroxine; Triac, 3,3',5-triiodothyroacetic acid; Tetrac,

624 3,3',5,5'-tetraiodothyroacetic acid; rT3, 3,3',5'-triiodo-L-thyronine, T2, 3,5-diiodo-L-thyronine, D-T3,

625 3,3',5-triiodo-D-thyronine, TIP, 2,4,6-triiodophenol, 2,4-DNP, 2,4-dinitrophenol.

626

627 Fig. 4. Effects of ethylenediaminetetraacetic acid (EDTA) and divalent cations on [^{125}I]T3 binding to

628 recombinant skate transthyretin (TTR). (*A*) Effect of divalent cations of [^{125}I]T3 binding to skate TTR. TTR

629 (0.53 nM) was incubated with 0.1 nM [^{125}I]T3 in the presence or absence (control) of 1 mM each divalent

630 cation for 1 h at 4°C. (*B*) Effect of pre-treatment of TTR at various concentrations with 1 mM EDTA on

631 [^{125}I]T3 binding. TTR (0.53–2640 nM) was pre-incubated with or without (control) 1 mM EDTA for 30 min

632 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

633 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

634 EDTA. TTR (26.4 nM) was pre-incubated with or without (control) 1 mM EDTA for 30 min for 4°C. After

635 dilution 50 times with the buffer (final concentration of EDTA = 0.02 mM), TTR (0.53 nM) was incubated

636 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

637 the TTR that was treated with neither EDTA nor cation (*EDTA-*, *cation -*) is indicated as the control (100%).

638 (*D*). Zn^{2+}-dependent activation of [^{125}I]T3 binding to TTR. TTR (26.4 nM) was pre-incubated with 1 mM

639 EDTA for 30 min for 4°C. After dilution 50 times with the buffer, TTR (0.53 nM) was incubated with 0.1

640 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

641 untreated with 1 mM EDTA (*open circle*) is indicated as control 100%. Each value is the mean ± standard

642 error of the mean of triplicate determinations. Experiments were repeated two or three times. Asterisks

643 indicate significant differences in T3 binding activity between the reference sample [cation (-) in *panel A*;

644 EDTA (-) in *panel B*; EDTA(+) and cation (–) in *panels C* and *D*] and each test sample. *, $p < 0.05$; **,

645 $p < 0.01$.

646

647 Fig. 5. Competition of iodothyronines and their related compounds for [^{125}I]T3 binding to recombinant

648 skate transthyretin (TTR). TTR (0.53 nM) was incubated with 0.1 nM [^{125}I]T3 for 1 h at 4°C, in the

649 presence or absence (control) of increasing concentrations of the following compounds:

650 3,3',5-triiodothyroacetic acid (Triac), T3, D-T3, 3,3',5,5'-tetraiodothyroacetic acid (Tetrac), T4, reverse T3

651 (rT3), 3,5-diiodo-L-thyronine (T2), 3,5-diiodo-L-tyrosine (Y2) and 3-iodo-L-tyrosine (Y1). Non-specific

652 binding, which was 3–4 % of total binding, was subtracted from total binding to give values for specific

653 binding. Each value is the mean of triplicate determinations. These experiments were repeated at least three

654 times.

655

656 Fig. 6. Inhibition of [^{125}I]T$_3$ binding to recombinant skate transthyretin (TTR) by various environmental

657 chemicals. (*A*) TTR (0.53 nM) was incubated with 0.1 nM [^{125}I]T3 in the presence of 1 μM of each

658 chemicals or solvent (dimethylsulfoxide, control) for 1 h at 4°C. Asterisks indicate significantly differences

659 in [^{125}I]T$_3$ binding activity between control and test samples containing each environmental chemical (**,

660 $p < 0.01$). (*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

661 (control) of increasing concentrations of the following compounds: pentabromophenol (PBP), triiodophenol

662 (TIP), pentachlorophenol (PCP), tetrabromobisphenol A (Br4-BPA), tetrachlorobisphenol A (Cl4-BPA),

663 ioxynil, tribromophenol (TBP) and trichlorophenol (TCP). To estimate the relative potency of the test

664 chemicals against T3, the competition curve for unlabeled T3 (broken line) was inserted. Non-specific

665 binding, which was less than 6% of total binding, was subtracted from total binding to give values for

666 specific binding. Each value is the mean of triplicate determinations. These experiments were repeated at

667 least three times.

668

669 Fig. 7. Scatchard plots of [125I]T3 binding to recombinant skate transthyretin (TTR). For high-affinity and

670 low affinity sites, TTR was incubated with 0.1 nM [125I]T3 M in the presence or absence of various

671 concentrations of unlabeled excess T3 (5 μM) in the incubation buffer with (●; 5.3 nM TTR) or without (○;

672 0.53 nM TTR) 1 mM EDTA for 1 h at 4°C. For high-affinity sites, TTR (0.53 nM) was incubated with

673 [125I]T3 ranging from 0.05 to 0.4 nM in the presence or absence of unlabeled excess T3 (5 μM) in the

674 incubation buffer for 1 h at 4°C (*inset*). Non-specific binding, which was 12–16% of total binding, was

675 subtracted from total binding to give values for specific binding. Each value is the mean of triplicate

676 determinations. These experiments were repeated three or four times. ν, molar ratio of bound [125I]T3 to

677 TTR concentration.

678

679 Fig. 8. Time course of enzyme-dependent 5-hydroxyisourate (HIU) hydrolysis by recombinant rainbow

680 trout 5-hydroxyisourate hydrolase (HIUHase) and recombinant skate transthyretin (TTR). HIUHase, TTR

681 and blank (buffer) were added to the reaction solution containing HIU (*arrow*). The hydrolysis of HIU was

682 monitored spectrophotometrically at 312 nm as described in the Materials and Methods. Enzyme-dependent

683 HIU hydrolysis indicates the difference in absorbance at 312 nm between the samples in the absence

684 (spontaneous degradation of HIU) and the presence of HIUHase or TTR (spontaneous + enzymatic

685 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 IC$_{50}$ for T3 to IC$_{50}$ 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/µg 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 ± 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.
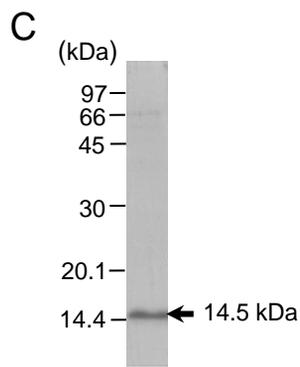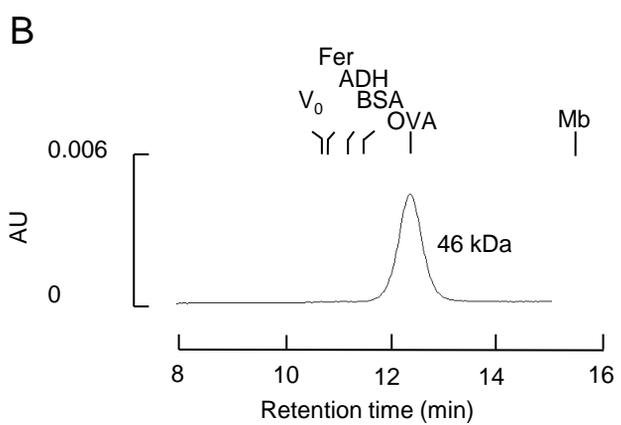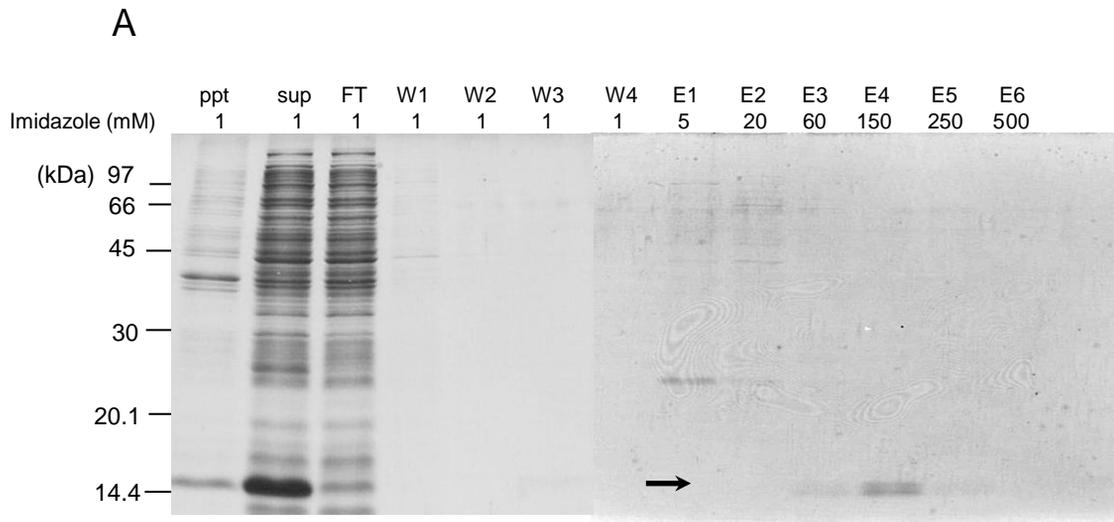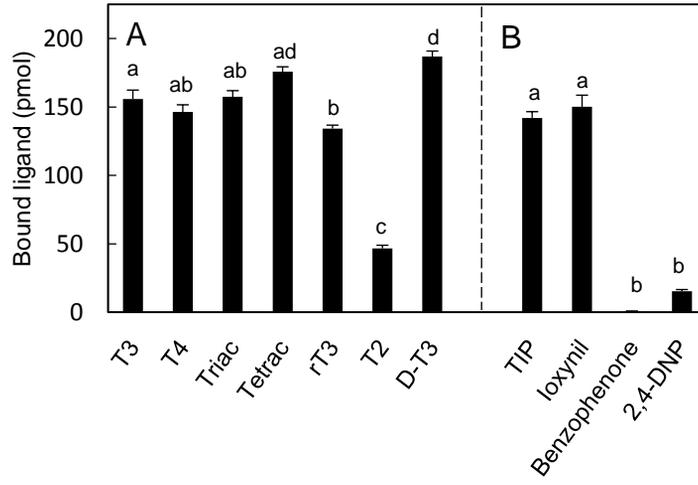
nd, not determined.

Fig. 1

```
                disordered          β-strand A        β-strand B      β-strand C    β-strand D
                <------------->     <-------->        <------->       <------->     <->
Lamprey    ADDDHKSHESHEGGVKDSCPLMVKAIDSVQGKPAAGVKLSVMKKQDDASWKEVATGVTGKTGESHHLISDKDFT
Skate          PHSHGDHHTRCPVLIKVLDALKGTPAANVQVQMLKRNEDKTWETINTGVTGGNGELHNLTSEEHLA
Trout         PVDRHGESDTHCPLMVKILDAVKGVPAGAVALSVSRRVNGMTWAQVASGVTDLTGEVHNLISDQDFQ
Seabream     TPTDKHGGSDTRCPLMVKILD-VKGTPAGSVALKVSQKTADGGWTQIATGVTDATGEIHNLITEQQFP
Toad        APPGHASHGEADSKCPLMVKVLDAVRGIPAANLLVNVFRQTESGKWEQITSGKTTELGEIHNLTTDEQFT
Bullfrog       THGEADSKCPLMVKVLDAVRGIPAAKLPVKVFKQNEDKSWDLISSGTTSSDGEIHNLATEEQFV
Crocodile   APLVSHGSIDSKCPLMVKVLDAVRGSPAANVAIKVFKKTSDGDWQEFAAGKTTEFGEVHELTSDEKFV
Chicken     APLVSHGSVDSKCPLMVKVLDAVRGSPAANVAVKVFKKAADGTWQDFATGKTTEFGEIHELTTEEQFV
Human        GPTGTGESKCPLMVKVLDAVRGSPAINVAVHVFRKAADDTWEPFASGKTSESGELHGLTTEEEFV
                   *      **    *    *     * **          *      * *    ** ** *
           ....|....|....|....|....|....|....|....|....|....|....|....|....|
              1        10        20        30        40        50        60
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           β-strand E   α-helix        β-strand F      β-strand G  β-strand H      No. of
           <------->    <------->       <------->       <------->   <------->       histidine
Lamprey    EGTYKVRFETQQYWTKTGITPFHEAAEVVFMAHGAGHKHYHIPMLLSPYFFATGAIVVDGKGH    11
Skate      VGLYKFHFETGAYWSNAGQAHFHECANVVFRVASLTSNHYTIAVLLSPYSYSTTGIIMNPHAT    11
Trout      SGVYRVEFDTKAYWKSQGTTPFHETAEVVFEAHAEGHRHYTLALLLSPFSYTTTAVVMKAHE    8
Seabream   AGVYRVEFDTKAYWTNQGSTPFHEVAEVVFDAHPEGHRHYTLALLLSPFSYTTTAVVSSVHE    7
Toad       EGVYKIEFATKAFWGKLGLSPFHEYVDVVFTANDAGHRQYTIAVLLTPYSFSSTAIVSEPHDDL   6
Bullfrog   EGIYKLEFATKRFWSKLGLTPFHEYVDVVFTANDAGHRHYTTAVLLTPYSFSTTAVVSDVKEAHV   6
Crocodile  EGIYRVEFDTSSYWKALGLSPFHEYADVVFTANDSGHRHYTIAALLSPFSYSTTAVVSDPQE    5
Chicken    EGVYRVEFDTSSYWKGLGLSPFHEYADVVFTANDSGHRHYTIAALLSPFSYSTTAVVSDPQE    5
Human      EGIYKVEILDTKSYWKALGISPFHEHAEVVFTANDSGPRRYTIAALLSPYSYSTTAVVTNPKE    4
              * *        *       *       ***    ***          *      ** *
           ....|....|....|....|....|....|....|....|....|....|....|....|....|
              70        80        90        100       110       120       130
```

Fig. 2

A

B

C

Fig. 3

Fig. 4

Fig. 5

Fig. 6

A

B

Fig. 7

Fig. 8

Fig. 5

Fig. 6

A

B