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Abstract The thyroid hormone binding protein transthyretin (TTR) forms a macromolecular complex with the retinol-specific carrier retinol binding protein (RBP) in the blood of higher vertebrates. Piscine TTR is shown here to exhibit high binding affinity for L-thyroxine and negligible affinity for RBP. The 1.56 Å resolution X-ray structure of sea bream TTR, compared with that of human TTR, reveals a high degree of conservation of the thyroid hormone binding sites. In contrast, some amino acid differences in discrete regions of sea bream TTR appear to be responsible for the lack of protein–protein recognition, providing evidence for the crucial role played by a limited number of residues in the interaction between RBP and TTR. Overall, this study makes it possible to draw conclusions on evolutionary relationships for RBPs and TTRs of phylogenetically distant vertebrates.

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Key words: Transthyretin; Retinol binding protein; Thyroid hormone; Protein–protein interaction; Crystal structure

1. Introduction

Transthyretin (TTR) and retinol binding protein (RBP) are plasma transport proteins involved in at least two molecular interactions. They bind physiologically small ligands, i.e. L-thyroxine/triiodo-L-thyronine and all-*trans* retinol, respectively, and form a moderately tight protein–protein complex. As a result of constraints imposed by these interactions, as well as of structural requirements, the three-dimensional structures of TTR and RBP have been found to be well preserved in vertebrate species distant in evolution, such as those of human and avian TTRs and RBPs [1–4]. TTR is composed of four identical subunits of approximately 14 kDa, consisting mainly of two four-stranded β-sheets, which are assembled around a central channel containing two nearly identical thyroid hormone binding sites. RBP is a monomeric protein of 21 kDa, which specifically transports all-*trans* retinol from liver storage sites to target cells. Mammalian holo-RBP circulates

in plasma almost entirely bound to TTR. Various lines of evidence indicate that the association of RBP with TTR serves to prevent filtration of the relatively small RBP molecule through kidney glomeruli. A protein conformational change induced by retinol release from holo-RBP [5] is known to lead to a reduced binding affinity, of possible physiological significance, between apo-RBP and TTR [5,6]. X-ray crystallographic studies have provided molecular details of the interactions of small size ligands with RBP [2,7] and TTR [8–10] and of the recognition between the two proteins [11,12].

RBP has long been known to be present in the blood of fish. However, at variance with RBP from higher vertebrates, it could be isolated from fish plasma only in the uncomplexed form [13,14]. The recent identification of piscine TTR [15,16] raises the question of the role of this protein in the transport of retinol-RBP and thyroid hormones. Here, we report on distinctive binding and structural properties of sea bream TTR.

2. Materials and methods

2.1. Materials

Human holo-RBP and TTR were purified from fresh plasma and quantified as described [6]. Holo-RBP purified from carp (*Cyprinus carpio*) plasma and a complete cDNA from sea bream (*Sparus aurata*) encoding TTR were kind gifts of A. Marchesani and D. Power, respectively. A complete cDNA from carp encoding TTR was kindly supplied by M. Aprea (the amino acid sequence of carp TTR, deduced from the nucleotide sequence, has been determined by Aprea, M., Morgado, I., Power, D., Gaetani, S. and Bellovino, D., to be published). L-Thyroxine was from Sigma.

2.2. Cloning, bacterial expression and purification of piscine TTRs

The region of the cDNA clone from sea bream corresponding to the mature TTR was polymerase chain reaction (PCR)-amplified under standard conditions, using MasterTaq DNA polymerase (Eppendorf) and two sequence-specific primers: a *Nde*I-tailed upstream primer (5'-CATATGCCCCCACCCCCACG-3') and a downstream primer (5'-GGATCAGAACATGAGAGAAACAAG-3'). The amplification product was inserted into the pGEM vector (Promega) to generate the intermediate vector pGEM-sea bream TTR. The restriction fragment obtained from *Nde*I digestion of plasmid pGEM-sea bream TTR was then ligated into the dephosphorylated *Nde*I site of the expression vector pET11b (Novagen). The region of the cDNA corresponding to the mature carp TTR was PCR-amplified under standard conditions, using Vent DNA polymerase (New England BioLabs) and two sequence-specific phosphorylated primers: a *Nde*I-tailed upstream primer (5'-CATATGCCAGTGGGTATTACGG-3') and a downstream *Bam*HI modified primer (5'-CGATGTGGATCCGTTGTATGTGG-3'). The amplification product was cloned into the *Pvu*II-digested and dephosphorylated vector pNEB193 (New England BioLabs) to obtain

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Abbreviations: TTR, transthyretin; RBP, retinol binding protein; rmsd, root mean square deviation

the intermediate plasmid pNEB-carp TTR. The restriction fragment obtained from *Bam*HI/*Nde*I digestion of plasmid pNEB-carp TTR was then ligated into the *Nde*I/*Bam*HI-digested and dephosphorylated pET11b expression vector (Novagen).

The expression of sea bream and carp TTRs in *Escherichia coli* BL21-CodonPlus-RIL (Stratagene) cells was induced by adding 1 mM isopropyl-1-thio- β -D-galactopyranoside, and after 4 h incubation at 28°C, cells were lysed by twenty 30 s bursts of sonication. Sea bream and carp TTRs were purified to homogeneity using a two-step procedure, comprising gel filtration (Sephacryl S-200, Amersham Biosciences, for sea bream TTR, and Biogel P60, Bio-Rad, for carp TTR) and anion exchange chromatography (Q Sepharose, Pharmacia Biotech), with a final yield of 2–2.5 mg/l of cell culture. The absorption coefficients $A_{1\%,1\text{cm}}$ of sea bream and carp TTRs at 280 nm were estimated to be 11.6 and 12.6, respectively, on the basis of their amino acid sequences.

2.3. Binding assays

The interaction of L-thyroxine with piscine TTRs was investigated by fluorescence titrations carried out with a Perkin Elmer LS-50B spectrofluorometer. Recombinant TTRs were supplemented with small aliquots of L-thyroxine, dissolved in 10 mM NaOH, and gently stirred in the spectrofluorometer cuvette. Ligand binding was monitored by measuring the quenching of the intrinsic protein fluorescence.

The most sensitive technique used so far to study in vitro the interaction between RBP and TTR is fluorescence anisotropy. The intensities of the vertical (I_v) and horizontal (I_h) fluorescence components were recorded at an angle of 90° to the vertically polarized excitation beam. A correction factor, G , equal to I'_v/I'_h , the primes indicating excitation polarized in a perpendicular direction, was used to correct for the unequal transmission of differently polarized light. Fluorescence anisotropy (A) is defined according to the equation: $A = (I_v - GI_h)/(I_v + 2GI_h)$. Human and carp holo-RBPs were titrated by adding aliquots of concentrated solutions of either human TTR or piscine TTRs to the RBP-containing samples, and the increase in fluorescence anisotropy of the RBP-bound retinol upon interaction with TTR was monitored.

2.4. Protein crystallization, data collection, and structure determination and refinement

Single crystals of sea bream TTR were obtained at 20°C in about 1 week, using the hanging drop vapor diffusion method, by equilibration with a solution containing 50 mM cadmium chloride, 50 mM sodium acetate, 15% (w/v) PEG 400, pH 4.6. Diffraction data were measured at the XRD1 beamline of Elettra synchrotron (Trieste, Italy) at cryogenic temperature. The detector used was a MAR CCD and the wavelength 1.2 Å. An entire data set at a maximum resolution of 1.56 Å was collected using one single crystal. One hundred and eighty frames with a crystal-to-detector distance of 80 mm and an oscillation of 1° and 45 frames with a distance of 150 mm and an oscillation of 2° were measured. The data indexing and integration were performed with the program Mosflm [17] and the scaling and merging with the program Scala [18]. The structure of sea bream TTR was solved by molecular replacement, with the program AmoRe [19]. The coordinates of the dimer of human TTR (PDB ID code 1F41 [8]) were used as a template. Because the crystals contain a tetramer in the asymmetric unit, the position of a dimer was found and, after fixing it, the second dimer was positioned. The residues of human TTR that are different relative to sea bream TTR were then replaced. Visualization of the model and manual rebuilding was performed with the program XtalView [20]. The first steps of refinement were performed with the program CNS [21], imposing a strict equivalence among the four protein subunits of the tetramer. The refinement was then completed to a final $R_{\text{factor}}/R_{\text{free}}$ of 19.7/22.3% by using the program SHELXL [22]. The quality of the final model was assessed using the program PROCHECK [23]. Stereochemical parameters are as expected for molecular models at this resolution. 91.6% of residues are in the most favored regions of the Ramachandran plot and 7.9% of residues in additionally allowed regions. In addition, H98 and P99 fall in the generously allowed and disallowed regions, respectively. It should be noted, however, that the latter residues are located in a disordered loop and they were only tentatively fitted in the electron density. The overall G factor is -0.1 . The complete statistics for data collection, processing and refinement are shown in Table 1.

Table 1
Statistics on data collection, processing and refinement^a

Space group	C2
Cell dimensions	$a = 96.452 \text{ \AA}$, $b = 65.622 \text{ \AA}$, $c = 70.834 \text{ \AA}$, $\beta = 97.4^\circ$, $z = 4$
Resolution (Å)	48–1.56 (1.64–1.56)
Total reflections/Unique	239 286/60 660
Overall completeness (%)	97.2 (89.9)
R_{sym} (%)	5.5 (14.6)
Multiplicity	3.9 (3.2)
$\langle I/\sigma(I) \rangle$	7.0 (2.1)
Total reflections used	60 408
Reflections in test set (10%)	6040
$R_{\text{cryst}}/R_{\text{free}}$	0.197/0.223
Protein atoms	3519
Solvent molecules/ions	365/2
rmsd on bonds (Å)/angles (°)	0.019/0.02

^aNumbers in parentheses refer to the last resolution shell.

3. Results and discussion

3.1. Amino acid sequence analysis

A multiple amino acid sequence alignment between TTRs of different vertebrates is presented in Fig. 1A. The amino acid sequence of sea bream TTR, deduced from the nucleotide sequence, shares identities of 51, 61 and 72%, respectively, with human, avian and trout TTR. As for the main residues of TTR known to be involved in the formation of the RBP–TTR complex in higher vertebrates [11,24], they are identical or chemically similar only partly in piscine TTRs. Specifically, in the amino acid sequences of the latter proteins, including that of carp TTR (not shown), the residues at positions 84, 99 and 100 are significantly different as compared to the TTRs of higher vertebrates (Fig. 1A). For a comparison, in the case of a multiple amino acid sequence alignment between RBPs of different vertebrates (Fig. 1B) the main residues of RBP known to be involved in the formation of the RBP–TTR complex in higher vertebrates [11,24] are identical or chemically similar in the presented sequences, except for residue 99, which is a T in piscine RBP and a K in the RBPs of higher vertebrates. It is worth noting that despite the fact that residue 95 is not fully conserved in all of the reported RBPs, its replacement does not appear to be particularly significant. In fact, residue 95 is an A in carp RBP, but is an S in sea bream, trout, mammalian and avian RBPs (Fig. 1B).

3.2. Binding properties of piscine TTRs

The heterologously expressed sea bream TTR is functionally active, as revealed by its ability to interact with L-thyroxine (Fig. 2A). The binding of L-thyroxine to TTR is known to produce a significant quenching of the intrinsic protein fluorescence. The titration shown in Fig. 2A was carried out using a rather high protein concentration (5 μM), a condition suitable for promoting stoichiometric binding of L-thyroxine and for the evaluation of the stoichiometry of binding of the hormone to the TTR molecule. Under these conditions, a virtually linear fluorescence quenching was found for a large part of the titration. The number of L-thyroxine binding sites per TTR molecule can thus be estimated from the intersection point of the linear decrease of fluorescence intensity and its plateau value at nearly saturating ligand concentrations. Since the intersection point corresponds to a L-thyroxine/TTR molar ratio of about two, this direct binding assay indicates the presence of two sites for L-thyroxine on piscine TTR. Similar

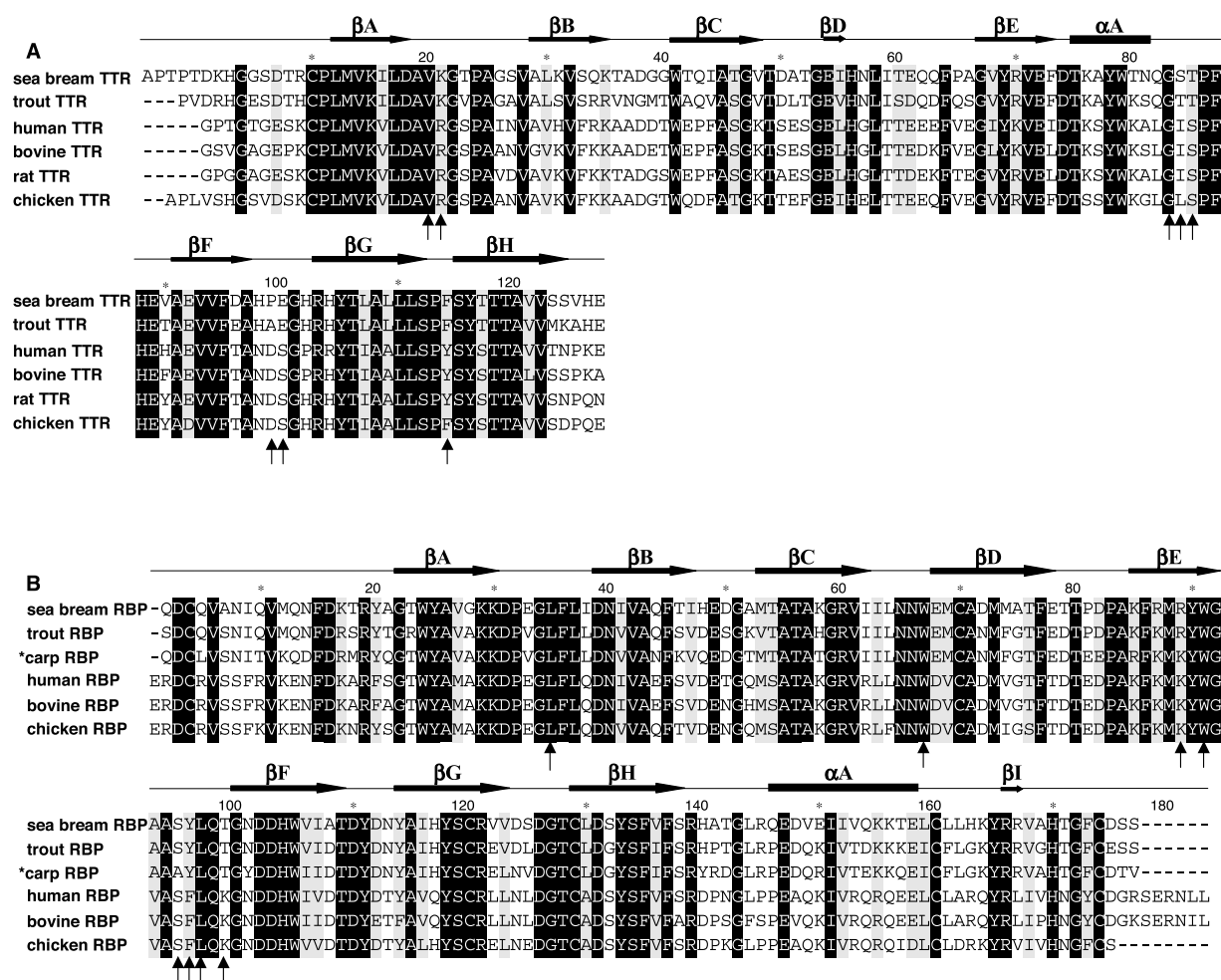


Fig. 1. Amino acid sequence alignments. A: Multiple amino acid sequence alignment for TTRs from different vertebrate species. B: Multiple amino acid sequence alignment for RBPs from different vertebrate species. Residues that are identical or chemically similar in TTR or RBP sequences are boxed in black or gray, respectively. Numbering and secondary structure elements are based on the structures of human TTR and RBP. The positions of β -strands and α -helices are denoted by arrows and boxes, respectively. Small vertical arrows below amino acid residues for both TTR and RBP sequences denote the position of main residues involved in TTR–RBP interactions in higher vertebrates, according to [11,24]. GenBank or SwissProt accession numbers are: trout TTR, CB497711 (EST sequence); human TTR, P02766; bovine TTR, NM_173967; rat TTR, NP_036813; chicken TTR, S17827; sea bream RBP, AAK49335; trout RBP, P24774; carp RBP, CAC12738; human RBP, P02753; bovine RBP, P18902; chicken RBP, P41263. *For the complete sequence of the N-terminus of carp RBP, see [25].

results were also obtained using recombinant carp TTR (data not shown).

We then addressed the question of whether piscine TTRs display binding affinity for RBP from either fish or mammals. The interaction with RBP of piscine TTRs was evaluated by analyzing the changes of the fluorescence anisotropy of RBP-bound retinol associated with the formation of the RBP–TTR complex, the molecular mass of which (76 kDa) is substantially higher than that of uncomplexed RBP (21 kDa). When sea bream or carp TTR was added to human holo-RBP, no significant increase in fluorescence anisotropy for the RBP-bound retinol was revealed under conditions suitable for the formation of the human TTR–RBP complex (Fig. 2B). Remarkably, a similar result was also obtained when sea bream or carp TTR was added to a piscine (carp) holo-RBP (Fig. 2C). No significant influence of the pre-incubation with L-thyroxine on the interaction of piscine TTR with RBP could be revealed when a titration was carried out in which sea bream TTR was incubated with saturating L-thyroxine prior to the addition to human holo-RBP (Fig. 2B). Therefore, sea bream

and carp TTRs appear to display a quite low affinity, if any, for RBP. It is interesting to note, however, that carp holo-RBP possesses binding affinity, albeit weak, for human TTR (Fig. 2C), a result consistent with the reported low affinity ($K_d = 15\text{--}40\ \mu\text{M}$) of two forms of trout RBP for human TTR [14]. Assuming a total change of fluorescence anisotropy similar to that found for mammalian RBP upon saturation by TTR, an apparent dissociation constant of $25\ \mu\text{M}$ can be estimated for the complex between carp RBP and sea bream TTR (Fig. 2C). In this respect, this value of the dissociation constant for the piscine RBP–human TTR complex must be compared with that of about $0.4\ \mu\text{M}$ for the homologous human RBP–TTR complex [26]. The above data are consistent with the lack in fish of the RBP–TTR complex, suggesting that it has evolved later in the evolutionary scale. Clearly, the lack of interaction between RBP and TTR in fish might cause a lowered retention of RBP in the circulation. However, as yet unidentified mechanisms preventing RBP from being cleared from the circulation might compensate for the presence of uncomplexed RBP in the blood of fish.

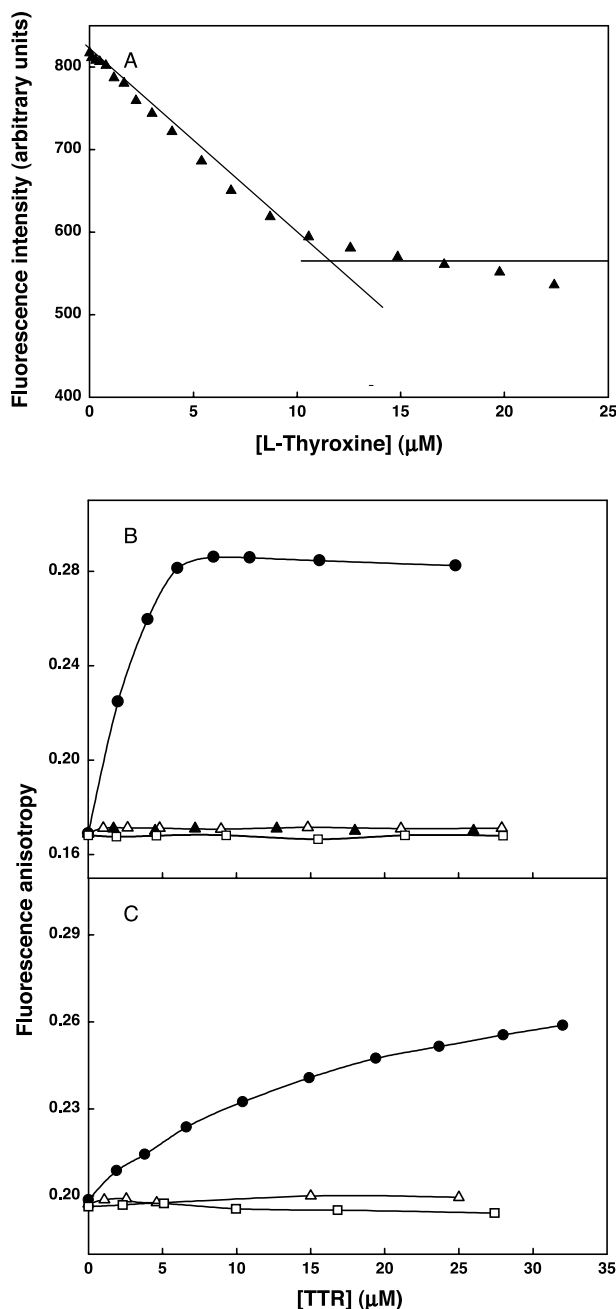


Fig. 2. A: Titration of sea bream TTR with L-thyroxine. Corrected intensity of protein fluorescence is plotted as a function of L-thyroxine concentration. Conditions were: 5 μM TTR in 0.05 M sodium phosphate, 0.15 M NaCl, pH 7.4, at $20 \pm 0.5^\circ\text{C}$. The quenching of the intrinsic protein fluorescence was monitored at 330 nm with excitation at 280 nm. B,C: Titrations of human and carp holo-RBPs with human, sea bream and carp TTRs. Fluorescence anisotropy values for either human (B) or carp (C) holo-RBP are plotted as a function of the concentration of either human TTR (●), sea bream TTR (Δ) or carp TTR (□). Human holo-RBP (B) was also titrated with sea bream TTR preincubated with a two-fold molar excess of L-thyroxine (▲). Conditions were: 9.3 μM holo-RBP in 0.05 M sodium phosphate, 0.15 M NaCl, pH 7.4, at $20 \pm 0.5^\circ\text{C}$. Retinol fluorescence was monitored at 460 nm with excitation at 330 nm.

3.3. Crystal structure of sea bream TTR compared with that of human TTR

The 1.56 Å crystal structure of sea bream TTR allows a good definition of the structural model (Fig. 3A). The electron

density map around the protein residues makes it possible to trace the polypeptide chain from residues 9 to 124, whilst those at the N- and C-termini are disordered, as also observed in the case of the human protein [8]. In the monoclinic crystal form we obtained an entire TTR tetramer is present in the asymmetric unit. In our model, 365 solvent molecules are visible and have been refined. Moreover, two penta-coordinated Cd^{2+} ions per TTR tetramer are bound in crystalline sea bream TTR (in this connection, it is worth recalling that Cd^{2+} ions were present in the crystallization medium) (Fig. 3A). The finding that only two Cd^{2+} ions are bound per TTR tetramer is not surprising, because of the presence of a tetramer in the asymmetric unit, which implies different intermolecular contacts for each subunit in the crystal. Each subunit of sea bream TTR was refined independently in the last refinement cycles, and a superposition of the four subunits gives a root mean square deviation (rmsd) for α -carbons ranging from 0.4 to 0.6 Å. Overall, the structure of sea bream TTR compares quite well with that of the mammalian proteins, such that the lack of binding affinity for RBP must rely on subtle structural differences affecting contact areas between RBP and TTR. The superposition of a dimer of sea bream TTR on that of human TTR (PDB ID code 1F4I [8]) is illustrated in Fig. 3B. The rmsd value between equivalent α -carbons is 0.6–0.7 Å, the largest values being found in correspondence of the loop 98–102, connecting β -strands F and C, which is considerably disordered in both piscine and human TTRs.

The superposition of the putative thyroid hormone binding sites of sea bream TTR on the corresponding L-thyroxine binding sites of human TTR (PDB ID code 1ict [9]) shows a nearly perfect structural correspondence for these binding sites present in the central channel of the TTR molecule. In fact, the examination of the side chains of the residues in proximity of bound L-thyroxine in human holo-TTR indicates a nearly perfect superposition with corresponding residues of sea bream TTR (Fig. 3C). The only exception is the S117T substitution in the piscine protein. However, this conservative substitution is unlikely to affect the interaction with thyroid hormones, on the basis of the structure of human TTR in complex with L-thyroxine [9]. Thus, structural data suggest similar thyroid hormone binding properties for piscine and human TTRs.

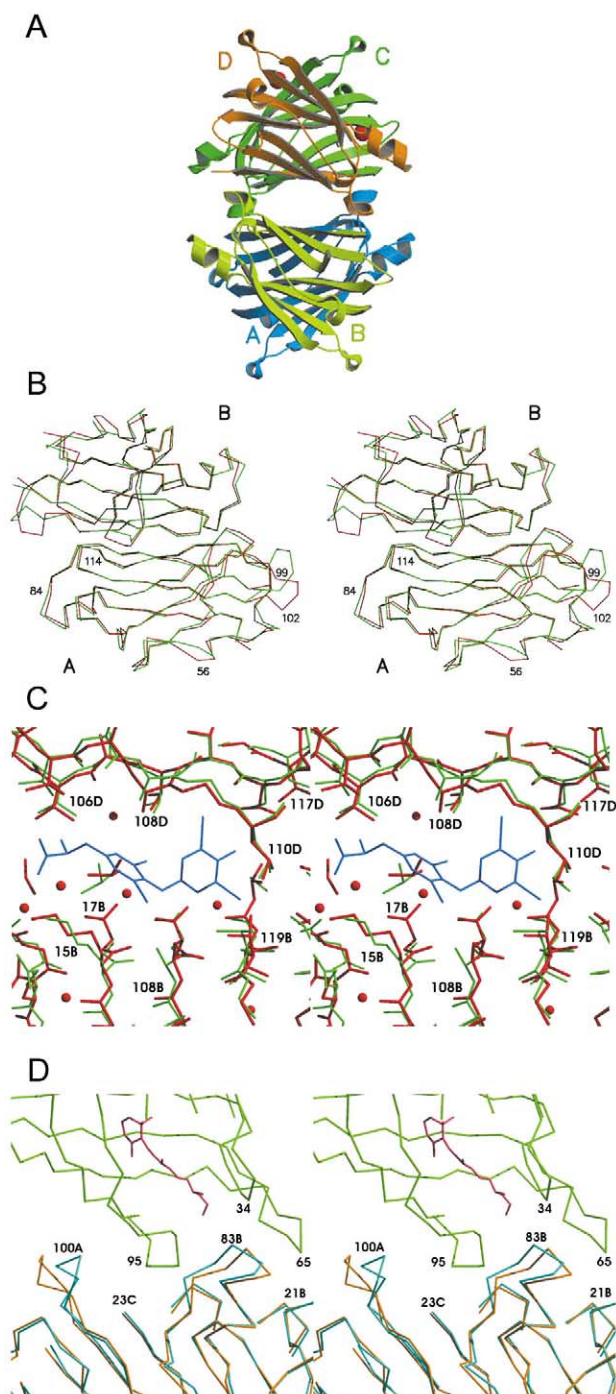
3.4. Structural basis for the negligible binding affinity of piscine TTR for RBP

The binding affinity of RBP and TTR of higher vertebrates is not particularly high ($K_d \approx 0.4 \mu\text{M}$ for the human RBP–TTR complex [26]) and a relatively limited number of contacts are established between interacting areas of the two proteins [11,24]. Therefore, the lack of some of these contacts is expected to drastically impair binding. A hypothetical model of a RBP–TTR complex containing sea bream TTR can be easily built superimposing the structure of the tetrameric sea bream TTR on that of human TTR present in the heterologous human TTR–chicken RBP complex (PDB ID code 1RLB [11]) (Fig. 3D). In the hypothetical model of the sea bream TTR–chicken RBP complex the portions of the C α chain trace of TTR in correspondence of two regions potentially interacting with RBP (loop 98–102 and region 81–84) assume different conformations compared to human TTR (Fig. 3D). In Table 2, the main residues of both RBP and TTR that are

in contact in the RBP–TTR complex, according to [11,24], are compared with equivalent residues present in sea bream RBP and TTR. It is evident that some of the residues present in sea bream TTR are significantly different relative to human TTR (Fig. 1A and Table 2): whereas two residues (V20 and G83) are fully conserved and three amino acid replacements (R21K, S85T and Y114F) are conservative, the three amino acid differences at positions 84, 99 and 100 can definitely perturb protein–protein interactions. The most significant amino acid differences are present in the loop 98–102: in sea bream TTR D99 is replaced by a P and S100 by an E. The former amino acid difference is particularly relevant, because the

partner in human/chicken RBP is the positively charged K99 and the corresponding possible partner in sea bream RBP is T99. Thus, the presence of an amino acid difference at position 99 in both sea bream TTR and RBP compared to human TTR appears to lead to the loss of a salt bridge in a hypothetical piscine RBP–TTR complex. Additionally, the presence of E instead of S at position 100 in sea bream TTR can lead to a rather unfavorable contact between E100 and the W91 partner present in human/chicken and sea bream RBP. The effect of the presence of S instead of I at position 84 in sea bream TTR is rather puzzling. In fact, it was observed previously that the interaction between human RBP and TTR is nearly abolished by the I84S replacement in human TTR [26]. The effect of this substitution was explained on the basis of a significant structural modification of the mutant form of TTR in the crystal, in which an H-bond interaction across the interface between two tetramers of TTR was observed [27]. In contrast, the above amino acid difference in sea bream TTR has no effect on the tetrameric organization of the protein. It should be noted, however, that the I84 residue present in two distinct subunits of the human TTR tetramer participates simultaneously in the interaction with one molecule of RBP (Table 2). Therefore, the substitution in TTR of a hydrophobic side chain (I) with a hydrophilic one (S) at position 84 leads to the loss of two significant contacts between residue 84 and two hydrophobic residues present in the RBP counterpart (Table 2). The loss of some interactions together with the effect of some unfavorable contacts is consistent with solution data indicating lack of binding, or at least a drastically reduced binding affinity, between piscine TTRs and RBP from both fish and mammals.

On the RBP side, the degree of conservation is much higher, because the only relevant amino acid difference in sea bream RBP relative to human/chicken RBP is K99T (Fig. 1B and Table 2), which is incompatible with the formation of the above mentioned salt bridge present in the RBP–TTR complexes of higher vertebrates. The higher degree of conservation of residues involved in the interaction between RBP and TTR is in keeping with the observation that piscine RBP is capable of interaction, albeit weak, with human TTR (Fig. 2C). Overall, these results indicate that the ability of RBP and TTR to interact with each other could be acquired during the course of evolution of vertebrates as a result



←
Fig. 3. Structure of sea bream TTR. A: Ribbon representation of the molecular model of sea bream TTR. The four subunits, labeled A–D, are shown in different colors. The central channel present in the TTR tetramer is the hormone binding site. The two red spheres represent two Cd^{2+} ions bound per TTR tetramer in the crystal. B: Superposition of dimers (α chain traces) of human (green; PDB ID code 1F41 [8]) and sea bream (red; this work) TTRs. C: Stereo representation of a portion of the central channel of sea bream TTR (red; this work) superimposed on the corresponding portion of the channel of human TTR (green; PDB ID code 1ict [9]) containing L-thyroxine (light blue). Solvent molecules (red spheres) are shown also. D: Stereo representation of the contact area of the RBP–TTR complex of higher vertebrates, with the superposition of the corresponding region of sea bream TTR. The model of the human TTR–chicken RBP complex (PDB ID code 1RLB [11]) was used as a template for the model of sea bream TTR present in a hypothetical protein–protein complex. Portions of α chain traces of chicken RBP (green), human TTR (light blue) and sea bream TTR (brown) are shown. The RBP-bound retinol molecule (red) is also shown.

Table 2

Comparison of the main amino acid residues in contact in the human/chicken RBP–human TTR complex [11,24] with corresponding residues of sea bream RBP and TTR^{a,b}

RBP (human/chicken)	RBP (sea bream)	TTR (human) ^c	TTR (sea bream)
L35	–	B-G83	–
W67	–	C-V20	–
W67	–	C-I84	S
K89	R	A-D99	P
W91	–	A-S100	E
S95	–	B-Y114	F
F96	Y	B-S85	T
F96	Y	B-Y114	F
F96	Y	B-I84	S
F96	Y	C-R21	K
L97	–	B-S85	T
K99	T	A-D99	P
K99	T	B-S85	T

^aResidues that are identical in sea bream RBP and TTR as compared to human RBP and TTR, respectively, are omitted.

^bAccession numbers for human, chicken and sea bream RBPs and for human TTR are reported in the legend of Fig. 1.

^cA, B and C: subunits of human TTR interacting with RBP [11,24].

of a few specific amino acid substitutions. The high degree of structural conservation of the thyroid hormone binding sites of sea bream and human TTRs and the strong effect of the replacement of a few amino acid residues on the affinity between RBP and TTR are consistent with the maintenance in all vertebrates of the function of TTR of binding thyroid hormones and with the occurrence of evolutionary changes affecting the ability of TTR to interact with RBP.

4. Structure deposition

Coordinates have been deposited at the Protein Data Bank as 1OO2 for release upon publication.

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