Transthyretin (TTR) is one of three proteins in the extracellular fluids of vertebrates responsible for the distribution of thyroid hormones and vitamin A. So far, TTR has only been found in vertebrates, of which piscine TTR displays the lowest sequence identity with human TTR (47%). Human and piscine TTR bind both thyroid hormones 3,5,3′-triiodo-L-thyronine (T3) and 3,5,3′,5′-tetraiodo-L-thyronine (thyroxine, T4). Human TTR has higher affinity for T3 than T4, whereas the reverse holds for piscine TTR. X-ray structures of Sparus aurata (sea bream) TTR have been determined as the apo-protein at 1.75 Å resolution and bound to ligands T3 and T4, both at 1.9 Å resolution. The apo structure is similar to human TTR with structural changes only at β-strand D. This strand forms an extended loop conformation similar to the one in chicken TTR. The piscine TTR-T3 complex shows the T3-binding site to be similar but not identical to human TTR, whereas the TTR-T4 complex shows the I3′ halogen situated at the site normally occupied by the hydroxyl group of T4. The significantly wider entrance of the hormone-binding channel in sea bream TTR, in combination with its narrower cavity, provides a structural explanation for the different binding affinities of human and piscine TTR to T3 and T4.

Complete cDNA sequence of a piscine transthyretin homologue was isolated from sea bream (Sparus aurata) and has 47% sequence identity with human TTR (5–7). Interestingly, piscine TTR displays a higher affinity for T3 than T4 (7, 8), as do the amphibian and avian TTRs. This is in contrast to the mammalian TTRs, which have higher affinity for T4 (9, 10). In birds, mammals, and mammals, TTR is expressed only in the liver and choroid plexus of the brain, whereas in reptiles it is expressed only in the choroid plexus. In fish or amphibians the main production is in the liver (2, 3, 11). This suggests that the binding affinities of TTR changed during evolution. In mammalian TTR, transport of T4 is preferred across the blood-brain barrier, where T4 is converted to the biologically more active T3 hormone (12). This evolution may correlate with the evolution of deiodinases, which generate T3 from T4 in a tissue-specific action (12, 13).

The three-dimensional structure of transthyretin is a homotetramer with a central hydrophobic channel in which two hormone-binding sites are located (14–17). The two retinol-binding protein-binding sites have also been structurally characterized and are situated on the surface of the molecule (18, 19). Previously determined structures of chicken, rat, and human TTR are very similar, with the exception of the α-helical region, which is somewhat different in the avian structure compared with the mammalian proteins (20, 21). Single point mutations in human transthyretin give rise to familial amyloidotic polyneuropathy (FAP), a hereditary form of amyloidosis associated with fibrillar TTR deposits in the peripheral nerves, kidney, spleen, heart, and eye (22–24). More than 70 different amino acid substitutions related to FAP have been identified so far (25), two of which are present in the sea bream TTR sequence, namely V30L and I84S (26). Structural changes within the TTR molecule underlie the formation of TTR amyloid fibrils (27, 28). To elucidate the mechanism behind TTR amyloidosis, structural analyses of TTR from other organisms can provide valuable information. This study describes three crystal structures: apo sea bream TTR at 1.75 Å resolution; in complex with T3 at 1.9 Å; and in complex with T4, also at 1.9 Å. The structures are compared with the human, rat, and chicken homologues, and molecular details behind thyroid-hormone binding are discussed.

MATERIALS AND METHODS

Cloning of Sea Bream TTR—A construct corresponding to the mature sea bream TTR, predicted by SignalP v1.1 (29), was amplified from the full-length cDNA located in a PA20H vector (7) using the forward primer 5′-TTT TTC ATG ACC CCC ACC CCC ACG-3′ (Interactive Virtual Laboratory). This primer introduces the N-terminal methionine and a BspH1 cleavage site, which changes the first Ala-19 codon GCC to ACC that encodes Thr. The reverse primer 5′-TTT CGA GCT CAC TCG TGT ACG CTG GAG-3′ (Interactive Virtual Laboratory) introduces a flanking SacI cleavage site following the C-terminal stop codon.

**High Resolution Crystal Structures of Piscine Transthyretin Reveal Different Binding Modes for Triiodothyronine and Thyroxine***

Received for publication, December 11, 2003, and in revised form, March 16, 2004

Published, JBC Papers in Press, April 13, 2004, DOI 10.1074/jbc.M313553200

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X-ray Structure of Sea Bream Transthyretin

After digestion with BspH1 and SacI (New England Biolabs/Amersham Biosciences), the fragment was introduced into a pET24d vector (kindly provided by Gunter Stier, EMBL-Heidelberg, Germany) cleaved with SacI and NcoI using the T4 DNA ligase Ready-To-Go kit (Amersham Biosciences). The ligated vector was used to transform competent E. coli BL21 cells with the pET24d vector containing the sea bream TTR gene. The plasmids were transformed (30) into E. coli BL21 cells with 30 µg/ml of water that included /H11002 the hanging-drop vapor-diffusion method (31). Drops with 8 µl of well solution containing 40 µg/ml pET24d vector were digested with BamH1 (New England Biolabs), whose cleavage site is provided by Gunter Stier, EMBL-Heidelberg, Germany cleaved with SacI and NcoI using the T4 DNA ligase Ready-To-Go kit (Amersham Biosciences) and an ABI 377 sequencer.

Table I: Data collection, refinement, and structural statistics for the sea bream TTR structures

| TTR (apo) | TTR/T3 | TTR/T4 |
|-----------|--------|--------|
| Data collection | | | |
| Space group | P41 | P41 | P41 |
| Cell dimensions (Å) | a = b = 58.50, c = 140.71 | a = b = 58.28, c = 138.90 | a = b = 58.46, c = 140.12 |
| Resolution (Highest resolution shell) (Å) | 30–1.75 (1.90) | 30–1.75 (1.90) | 30–1.75 (1.90) |
| Number of observations | 928494 | 305833 | 39592 |
| Unique reflections | 461044 (4416) | 360323 (3581) | 35496 (3244) |
| Completeness (%) | 97.1 (92.2) | 98.8 (96.6) | 96.0 (89.7) |
| Rmerge (%) | 4.7 (45.1) | 5.4 (42.4) | 4.7 (44.6) |
| Refinement | | | |
| Resolution range used in refinement (Å) | 20–1.75 | 20–1.9 | 20–1.9 |
| Reflections in working set | 39742 | 31100 | 30446 |
| Reflections in test set | 21.0 | 21.1 | 21.6 |
| Mean temperature factor (Å²) | 21.6 | 14.4 | 14.6 |
| R.m.s.d. bond lengths (Å) | 0.008 | 0.010 | 0.009 |
| R.m.s.d. bond angles (°) | 1.30 | 1.46 | 1.40 |
| Torsion angles period 1 (°) | 3.96 | 4.01 | 4.04 |
| Torsion angles period 3 (°) | 18.39 | 18.30 | 18.30 |

The hormone complexes diffracted to 1.75 Å resolution. Crystals of the two TTR isoforms diffracted to 1.9 Å resolution. Both complexes were crystallized in a similar fashion, i.e., the apo protein was incubated for 24 h at 4 °C with a saturated solution of T3 or T4 in 50 mM Tris, pH 7.5, prior to the crystallization trials. All data sets were collected at 100 K from single crystals mounted in nylon loops (32), using synchrotron radiation at beam line X11, European Molecular Biology Laboratory outstation, German Synchrotron Research Centre, Hamburg, Germany. The data were processed and scaled with the programs DENZO and SCALEPACK (33), after which the intensities were converted to structure factors using the programs TRUNCATE and CAD from the CCP4 package (34). Data collection statistics are included in Table I.

Molecular Replacement and Structure Refinement—The structure of the apo protein was solved by molecular replacement using the program CNS (35). A poly(alanine) model of the human transthyretin tetramer was used as starting model (Protein Data Bank code 1F41, Ref. 16) with the BC loops (residues 36–40), CD loops (residues 50–52), a-helices (residues 80–86), and PG loops (residues 98–104) removed. The progress of refinement was followed by monitoring the Rmerge value from the start, with 10% of the reflections included in the test set (36). After rigid body refinement, nearly all side chains could be manually built into the first 2 Fobs–Fcalc map using the graphics software O (37). The structure was refined by subsequent positional and temperature factor refinement using CNS (35), REFMAC (38), and manual model building. The hormone complexes were refined with REFMAC (38) by means of difference Fourier techniques using the structure of the piscine apo protein. The coordinates for T3 and T4 were derived from the Hetero compound Information Centre (39). The refined models comprise good agreement factors using the programs TRUNCATE and CAD from the CCP4 package (34). Data collection statistics are included in Table I.

The Structure of Piscine Transthyretin—The structure of sea bream TTR was solved using molecular replacement methods with a truncated poly(alanine) model of human TTR (PDB accession code 1F41, Ref. 16) as a search model. The quality of the initial electron density map was excellent and allowed most side chains of the fish TTR structure to be modeled. The four monomers (A, B, C, and D) comprising the tetramer in the asymmetric unit were refined independently of each other from the start. The final model includes residues A10–A127, B10–B125, C11–C125, D10–D123, 361 water molecules, and one sulfate ion (Fig. 1). In some cases, additional electron density was observed at the N- and C-terminal ends, but the quality did not allow additional residues to be exclusively positioned. The side chains of residues A-Gln-63, C-Glu-61, C-Gln-62, C-His-102, C-Ser-124, D-Thr-119. The 361 water molecules were positioned within the initial electron density map using the graphics software O (37). The coordinates for T3 and T4 were derived from the Hetero compound Information Centre (39). The refined models comprise good agreement factors using the programs TRUNCATE and CAD from the CCP4 package (34). Data collection statistics are included in Table I.

RESULTS AND DISCUSSION

The Structure of Piscine Transthyretin—The structure of sea bream TTR was solved using molecular replacement methods with a truncated poly(alanine) model of human TTR (PDB accession code 1F41, Ref. 16) as a search model. The quality of the initial electron density map was excellent and allowed most side chains of the fish TTR structure to be modeled. The four monomers (A, B, C, and D) comprising the tetramer in the asymmetric unit were refined independently of each other from the start. The final model includes residues A10–A127, B10–B125, C11–C125, D10–D123, 361 water molecules, and one sulfate ion (Fig. 1). In some cases, additional electron density was observed at the N- and C-terminal ends, but the quality did not allow additional residues to be exclusively positioned. The side chains of residues A-Gln-63, C-Glu-61, C-Gln-62, C-His-102, C-Ser-124, D-Gln-62, and D-Gly-101 displayed weak electron density. Seven residues were refined in two well defined conformations: A-Ser-84, A-Asp-96, B-Ser-84, B-Thr-119, C-Asp-96, C-Thr-119, and D-Thr-119. The 361 water molecules were positioned within
The four monomers in sea bream TTR are very similar. Root mean square deviations comparing the main chain Ca atoms of residues 12–123 among all monomers varies from 0.27 Å (comparing monomers A and B) to 0.51 Å (comparing monomers A and C). The sulfate ion bridges the main chain carbonyl oxygen of His-102 in the FG loop of monomer A and the Oy atom of Ser-123 to the main chain nitrogen atom of Ser-124 at the C-terminal end of monomer A. This sulfate ion is also part of the crystal-packing interface forming a salt link to the side chain of Arg-103 in a symmetry-related copy of monomer B (B'). All sulfate atoms are well defined with B-factors of 34 Å² and restrict the mobility at the C-terminal end of monomer A, which could be modeled to the last residue, A-Glu-127.

The N terminus of piscine, avian, and reptilian TTR has three additional hydrophobic amino acids, DKH, compared with the eutherian proteins. A chimeric molecule generated from crocodile TTR, in which the N-terminal end was replaced by that from frog, was shown to differ in both the specificity and affinity of T₄ and T₃ binding (13). Interestingly, in the sea bream structure, electron density runs through the center of the hormone-binding channel visible at lower contour levels of the electron density maps (Fig. 2). We could not identify the source of this density, but water molecules seem unlikely because the density does not connect to potential hydrogen-bonding partners on the protein. Two possibilities are that the electron density corresponds to residues at the N-terminal end of the protein or to polyethylene glycol molecules present in the crystallization medium.

While this work was in revision, a structure of sea bream TTR in a different crystal form was independently reported by Folli et al. (44). We find their structure virtually identical to ours with the exception of the positions of the FG loops. There have been controversies about the correct amino acid at position 103 (5, 7). By sequencing the clone, we would like to correct our original data published in Ref. 7 and confirm that the correct amino acid at position 103 should be an arginine. It should, however, be noted that the structure deposited by Folli et al. is reported to have a glycine at this position.

Comparison of Piscine TTR with Other Species—Crystal structures of TTR are available from three other species: human (16), chicken (20), and rat (21). Overall, the structure of sea bream TTR is very similar to those of rat and human with root mean square deviations of 0.62 and 0.71 Å, respectively, when superimposed on residues 12–98 and 104–123. Chicken TTR deviates significantly from fish, human, and rat in the conformation of the α-helix and succeeding EF loop (residues 75–88), and its root mean square deviation to sea bream TTR is 1.3 Å. These differences are most likely caused by a sulfate ion located in the α-helix region of the chicken structure, a different position compared with the sulfate found in sea bream TTR, that binds to the FG-loop (residues His-102 and Arg-103) and C-terminal (residues Ser-123 and Ser-124).

Water molecules W1-W36 play a significant role in stabilizing the monomeric (W1-W12), dimeric (W13-W20), and tetrameric (W21-W36) forms of the human protein (16). These water molecules are not strictly conserved in sea bream TTR, but all the corresponding molecules were numbered equivalent to human TTR (W1-W36 in monomers A and B and V1-V36 in monomers C and D) (Fig. 1). One novel water molecule is situated at the edge-strand region of each monomer, which is the only area with significant structural differences between human and piscine TTR. When comparing the two structures, the dissimilarity is already visible at the end of β-strand B, even more so in β-strand C, and most apparent in what corre-
that only six familial amyloidotic polyneuropathy mutations occur as natural amino acids in other species (4). Leu-68 is found in eight species, Thr-45 in African clawed frog, Asp-42 and Ser-45 in bullfrog, and Leu-30 and Ser-84 in fish. The positions of both Leu-30 and Ser-84 are well defined in the electron density of sea bream TTR, but their structural significance is difficult to interpret because they are situated in regions with additional side chain substitutions compared with the human protein.

**Hormone Binding to Sea Bream TTR**—We have previously shown that the binding properties of T3 and T4 for purified recombinant sea bream TTR are similar to those for the endogenous serum protein in sea bream (4). The x-ray crystal structures of human TTR in complex with T3 provide the first detailed description of a thyroid hormone binding to TTR (17, 45). These studies show that the two TTR hormone-binding sites, situated between monomers A, C and B, D, respectively, can be divided in three sections: an inner and outer cavity comprising three symmetry-related pairs of halogen-binding pockets, HBP1 (HBP1′), HBP2 (HBP2′), and HBP3 (HBP3′). The 2-fold symmetry axis of the TTR homotetramer runs in the direction of the hormone-binding channel and coincides with the 2-fold crystallographic symmetry axis in crystals with the P21212 space group. However, most TTR ligands lack 2-fold symmetry, and subsequently the x-ray structures of human TTR complexes in the P21212 space group have to be refined with the ligand positioned in two symmetry-related conformations with 50% occupancy. This complicates the electron density map interpretation and reduces the accuracy of the model. Recently, aligned thyroxine molecules were presented from structural studies of human and rat TTR crystals with tetramers rather than dimers in the asymmetric unit (46, 47). Interestingly, these structures show that ligand binding imposes subtle structural differences in the AC dimer compared with the BD dimer that is selected for in the crystal packing of the TTR tetramers. A multitude of structural data about TTR-ligand complexes is available (17, 45, 47–53). Combined, these studies show a complicated picture of TTR-ligand interactions where the same ligand can have more than one binding site in the hormone-binding channel.

T3 comprises four iodines: I3 and I5 at the tyrosyl ring and I3′ and I5′ at the phenolic ring. In the human TTR-T3 complex, the I3 and I5 iodines are situated at the outer HBP1 site, whereas I3′ and I5′ are situated at the innermost HBP2 and HBP3 sites (17, 45). The polar amino group interacts with the charged residues Lys-15 and Glu-54 at the entrance of the channel. In the structure of T4 in complex with rat TTR, T4 occupies two positions in the AC and BD-binding sites of the tetramer. One position is similar to T3 in the human protein where the phenolic iodines occupy the HBP2/HBP3′ pocket; in the second position the phenolic iodines occupy the HBP3/HBP3′ pocket (47). The structure of human TTR in complex with T3 is not known. We have made several attempts to obtain this structure but so far without success. We have collected high resolution diffraction data of both crystals soaked in T3 and of co-crystals obtained at high concentration of T3. In both cases, the determined protein structures represented the apo form of the protein. We did not detect any binding of T3 to human recombinant TTR in the dot-blot analysis, which shows strong binding of T3 to sea bream TTR (4). This suggests that binding of T3 to human recombinant TTR is weaker than to the serum TTR protein.

The sea bream TTR complexes with T3 and T4 were each studied at 1.90 Å resolution. Generally, hormone binding did not induce any major structural changes and strand D maintained its extended loop conformation. The position of the three and four iodines in T3 and T4, respectively, could be clearly identified from omit maps (Fig. 4). However, the thyronine rings and alanyl moiety are less well defined in both complex structures. As in the

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**Fig. 3.** The conformation of β-strand D in monomer A. A, human TTR; B, sea bream TTR. β-strand D, defined by three hydrogen bonds from residues Gly-53 and Leu-55 to residues Val-16 and -14 situated on β-strand A, are indicated as black dashed lines in the human structure. The novel water molecule W39 in sea bream is pink; waters W9 and W10 present in both structures are green.

**Fig. 4.** The crystal packing of the TTR tetramer. A multitude of structural data about TTR-ligand complexes is available (17, 45, 47–53). Combined, these studies show a complicated picture of TTR-ligand interactions where the same ligand can have more than one binding site in the hormone-binding channel.
previously described human and rat TTR tetramers, the electron density maps display one unique binding mode of the T4 hormone, suggesting that sea bream TTR tetramers are aligned within the crystals. T4 binds similarly to sea bream TTR compared with human and rat TTR. However, the hormone molecule does not reach as deep into the hydrophobic channel of sea bream TTR as it does in human and rat TTR; thus, none of the four iodines binds to the HBP3. C, the sea bream TTR-T4 complex. Three strong electron density features correspond to two equivalent positions of the T4 ligand. The fourth and weaker electron density feature shows that a smaller fraction of the T4 molecules is position equivalent to the T4 molecule. This density is, however, not present in monomer B of sea bream TTR. D, comparison of T4 (dark blue) and T3 (red) binding to sea bream TTR. The sea bream complex structures in panels A and C are colored as in Fig. 2. Omit \( F_o - F_c \) maps are contoured at 6 \( \sigma \) over the hormone-binding site.

Fig. 4. T3 and T4 binding in monomer A of sea bream TTR (A) of the sea bream TTR-T4 complex. B, T4 binding to human TTR (light gray), rat TTR (blue), and sea bream TTR (dark blue). All structures are superimposed on residues A12-A98 and A104-A123. The T4 molecule does not reach as deep into the hydrophobic channel of sea bream TTR as it does in human and rat TTR; thus, none of the four iodines binds to the HBP3.
HBP2 and HBP2'. Furthermore, the HBP2 and HBP2' sites do not bind iodine in the piscine TTR-T complex.

In the TTR-T complex, four strong electron density features were observed in the AC-binding site, one of which is situated at the position of the hydroxyl group, O4', in T4. This site is not occupied to a large extent by the third iodine, I3', in T4 (Fig. 4C). In this novel T3 conformation, the O4' group is instead occupied to a large extent by the third iodine, I3' in T4 complex. Further structural studies are needed to elucidate whether HBP3 of piscine TTR replaces the hydroxyl group in the human TTR.

With the exception of the number of water molecules within the apolar regions, the hormone-binding channels of sea bream and human TTR are similar overall. In addition, the hormone-binding channels of sea bream and human TTR differ in their size, which is evident upon comparing the distance between two Glu-54 atoms (19.0 Å) in sea bream and human TTR. However, the shape of the hormone-binding channels of sea bream and human TTR is different, which explains the discrepancies in T3 and T4 binding affinities of the two species. The areas of the cavity enclosing the inner phenolic and the outer tyrosyl rings of the hormone are clearly narrower in fish TTR. This is evident upon comparing the distance between two symmetry-related residues at position 108, which is 11.2 Å in sea bream TTR and 12.0 Å in human TTR, and between the Glu-54 atoms of two symmetry-related T4/15 residues. 12.5–12.6 Å in fish TTR compared with 13.5–13.8 Å in human TTR. In addition, the sea bream hormone-binding channel is wider at its entrance, right at the binding site for the alanyl group of the hormone. At the entrance, the distance between two CO2 atoms of Glu-54 is 18.9–19.0 Å in sea bream TTR compared with 17.5–17.6 Å in the human protein. These dissimilarities are due in part to a number of amino acid substitutions in the hydrophobic core of fish TTR compared with the human protein, including Ile-16 for Val, Leu-30 for Val, Ile-44 for Phe, Ile-55 for Leu, Ile-59 for Thr, Phe-73 for Ile, Leu-109 for Ala. Furthermore, different side chain rotamers of Val-14 and Leu-58 affect the position of the otherwise conserved residues involved in hormone binding. The significantly wider entrance of the hormone-binding channel in combination with its narrower inner and outer cavity provides a structural explanation for the different binding affinities of T3 and T4 to avian and piscine TTR compared with human TTR. The inner phenolic ring of the T3 molecule has only one iodine and is therefore smaller than the phenolic ring of T4, and in its novel position in the outer tyrosyl ring is parallel to the Lys-15 side chains in a conformation optimal for the restricted channel of sea bream TTR. Avian TTR, which also has higher affinity for T3 than T4 (10), displays a similar overall shape as sea bream TTR.
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J. Biol. Chem. 2004, 279:26411-26416.
doi: 10.1074/jbc.M313553200 originally published online April 13, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M313553200

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