The evolutionary and integrative roles of transthyretin in thyroid hormone homeostasis

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Abstract

In larger mammals, thyroid hormone-binding plasma proteins are albumin, transthyretin (TTR) and thyroxine (T4)-binding globulin. They differ characteristically in affinities and release rates for T4 and triiodothyronine (T3). Together, they form a 'buffering' system countering thyroid hormone permeation from aqueous to lipid phases. Evolution led to important differences in the expression pattern of these three proteins in tissues. In adult liver, TTR is only made in eutherians and herbivorous marsupials. During development, it is also made in tadpole and fish liver. More intense TTR synthesis than in liver is found in the choroid plexus of reptilians, birds and mammals, but none in the choroid plexus of amphibians and fish, i.e. species without a neocortex. All brain-made TTR is secreted into the cerebrospinal fluid, where it becomes the major thyroid hormone-binding protein. During ontogeny, the maximum TTR synthesis in the choroid plexus precedes that of the growth rate of the brain and occurs during the period of maximum neuroblast replication. TTR is only one component in a network of factors determining thyroid hormone distribution. This explains why, under laboratory conditions, TTR-knockout mice show no major abnormalities. The ratio of TTR affinity for T4 over affinity for T3 is higher in eutherians than in reptiles and birds. This favors T4 transport from blood to brain providing more substrate for conversion of the biologically less active T4 into the biologically more active T3 by the tissue-specific brain deiodinases. The change in affinity of TTR during evolution involves a shortening and an increase in the hydrophilicity of the N-terminal regions of the TTR subunits. The molecular mechanism for this change is a stepwise shift of the splice site at the intron 1/exon 2 border of the TTR gene. The shift probably results from a sequence of single base mutations. Thus, TTR evolution provides an example for a molecular mechanism of positive Darwinian evolution. The amino acid sequences of fish and amphibian TTRs are very similar to those in mammals, suggesting that substantial TTR evolution occurred before the vertebrate stage. Open reading frames for TTR-like sequences already exist in Caenorhabditis elegans, yeast and Escherichia coli genomes.

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Basic physico-chemical features of thyroid hormones important for their physiological function

Iodine content

The iodine atom stands out among all atoms in animal tissues by its large atomic weight (126·91 compared, for example, with 12·01 for carbon, 1·008 for hydrogen, and 16·000 for oxygen). The two thyroid hormones in the human body contain three (in triiodothyronine (T3)) or four iodines (in thyroxine (T4)) per molecule. The large size (a covalent radius of 0·133 nm for iodine, compared with 0·077 nm for carbon, 0·030 nm for hydrogen and 0·066 nm for oxygen) and the electronegativity of the iodine atoms in the thyroid hormones are important features for the specific interaction with and binding to proteins, features very appropriate for a ‘signal’ compound.

Solubility

Thyroid hormones are typical extracellular hydrophobic signaling molecules. In the bloodstream of larger mammals they are bound to the plasma proteins T4-binding globulin (TBG), transthyretin (TTR) and albumin (Larsson et al. 1985). This binding to plasma proteins is sometimes misinterpreted as ‘making soluble’ of the hormones for transport in the bloodstream (Alberts et al. 1994). Although being ‘relatively insoluble’ in water, the solubility of
Molecular cloning of rat TTR mRNA, tissue distribution of TTR mRNA, and regulation of TTR gene expression in brain and liver

Extending studies on the mechanism of the acute phase response of plasma protein synthesis in the liver (Dickson et al. 1982), rat liver mRNA was transcribed into cDNA and TTR cDNA was cloned and sequenced (Dickson et al. 1985a,b). The deduced sequence of 127 amino acids was 83% identical with that reported for human TTR by Kanda et al. (1974). For determining the lower threshold of detection, a tissue not expected to synthesize plasma proteins, namely brain, was selected as a negative control for TTR mRNA analysis by dot blot hybridization (Dickson et al. 1985a,b). Surprisingly, 1 g brain tissue contained about 7% of the amount of TTR mRNA found in 1 g liver. It seemed unlikely that a particular, specific function, such as TTR synthesis, would be distributed evenly throughout the brain. From histological (presence of cuboid, epithelial cells with ciliae and well developed Golgi apparatus and rich rough endoplasmic reticulum) and functional considerations (prominent position in influencing homeostasis in the extracellular compartment), the choroid plexus in the ventricles seemed to be the most likely place for TTR gene expression in the brain. Direct RNA extraction from choroid plexus and analysis confirmed this assumption (Dickson et al. 1985a,b). In situ hybridization showed that, apart from a very weak signal over the meninges, all of the brain TTR mRNA was located in the choroid plexus (Stauder et al. 1986). About 12% of protein newly synthesized by choroid plexus pieces incubated in vitro and about 43% of protein secreted into the medium was TTR (Dickson et al. 1986). TTR gene expression in the choroid plexus was constitutive, in contrast to the negative acute phase regulation of TTR synthesis in the liver (Dickson et al. 1986).

Northern analysis and dot blot hybridization are not very precise methods of mRNA quantitation. Therefore, hybridization with TTR cDNA in solution and ribonuclease protection were used for the molecular titration of TTR mRNA in liver and brain from chicken (Duan et al. 1985a,b).
1991) and rat (Schreiber et al. 1990). Tritiated authentic RNA served as an internal standard to correct for RNA losses during extraction and purification. The values obtained for the molecular TTR mRNA content of rat and chicken liver and choroid plexus are summarized in Table 1. The level of TTR mRNA was far higher in the choroid plexus than in the liver for both the chicken and the rat.

**Significance of TTR synthesis in the brain**

Experiments perfusing isolated sheep brains showed that all newly synthesized TTR was secreted from the choroid plexus towards the ventricles (Schreiber et al. 1990). For the human, it has been calculated that only 3% of TTR in the ventricular cerebrospinal fluid (CSF), and only 10% of the TTR in lumbar CSF, are derived from blood (Reiber 2001). A choroid plexus model could be developed in which a monolayer of rat choroid epithelial cells growing on a laminin-coated filter (which separated a lower and an upper chamber) synthesized and then secreted TTR unidirectionally into the upper chamber. Introduced radioactive T4 was found to accumulate in the upper chamber. This accumulation was prevented by inhibiting protein synthesis with cycloheximide (Southwell et al. 1993), suggesting that ongoing protein synthesis in the choroid plexus cells was necessary for the transport of T4 to the CSF side of the plexus.

Based on these experiments, the hypothesis illustrated in Fig. 2 was proposed. According to this hypothesis, TTR is synthesized in the choroid plexus epithelial cells and secreted towards the ventricle (uppermost cell in Fig. 2). In the CSF filling the ventricle, TTR binds T4. Alternatively, newly synthesized TTR could bind T4 already in the choroid plexus cell and the TTR-T4 complex is secreted into the CSF (choroid plexus cell in the middle of Fig. 2). Finally, some TTR may be synthesized and secreted into the CSF and not bind T4 at all near the choroid plexus (choroid plexus cell at the bottom). T4 is also likely to permeate through the blood–brain barrier away from the ventricular region (lower part of Fig. 2). This TTR will of course participate in equilibration between free and TTR-bound T4.

**TTR null mutants and biological importance of redundancy**

TTR null mutants have been created in mice (Episkopou et al. 1993). They were found to possess normal levels of free T4 in the blood, that is they were euthyroid (Palha et al. 1994). Mutations which lead to the absence of the thyroid hormone-binding protein, but which are associated with a normal phenotype are also known for albumin in humans (Kallee & Ott 1992) and rats (Mendel et al. 1989). Various TBG deficiencies have been observed in

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**Table 1** Concentration of TTR mRNA in choroid plexus and liver from rat and chicken. Method: ribonuclease protection assay + in-solution hybridization and correction for losses during extraction by prior addition of a calibrated standard of tritiated authentic TTR mRNA. Data for rat from Schreiber et al. (1990) and for chicken from Duan et al. (1991)

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**Figure 2** Distribution of TTR and T4 in blood-choroid plexus epithelial cells–CSF. Upper diagram represents choroid plexus epithelial cells, i.e. the blood–CSF barrier. Lower diagram represents the blood–brain barrier. The choroid plexus cells possess microvilli at the brain (CSF) side. All TTR synthesized in the choroid plexus is secreted towards the brain (Schreiber et al. 1990). TTR can either be synthesized and secreted into the CSF, where it binds T4 (uppermost cell), or it binds T4 already intracellularly and the complex TTR-T4 is secreted towards the brain (second cell from the top). Some TTR will be secreted without contact to T4 within the cell (third cell from top), flow through the ventricles and establish equilibrium with T4 later in the ventricular system. TTR can also permeate into and through the cells of the blood–brain barrier throughout the brain (lowest two cells). From Southwell et al. (1993), with permission © The Endocrine Society.
Overlapping binding characteristics of thyroid hormone-binding proteins

Affinities and on- and off-rates for the binding or release of thyroid hormones and proteins differ for TBG, TTR and albumin. This is illustrated for the proteins from humans in humans (Peizhi et al. 1991, Carvalho et al. 1998a, b, Carrel & Allen 1999, Reutrakul et al. 2001). In the absence of clinical evidence of hypo- or hyperthyroidism, they are often uncovered by the fortuitous finding of abnormally high or low concentrations of total T4 in blood (Mori et al. 1990).

Normal levels of corticotropin-releasing hormone (CRH), and even its increase in response to stress, were observed in null mutants of another hormone-binding protein, namely CRH-binding protein (Karolyi et al. 1999).

All these observations strongly suggest that these systems of hormone-binding proteins involve several functionally redundant components. Such redundancy is likely to provide a strong advantage in natural selection: one deficient component of the system can be compensated for by the other components involved. Thus, Palha et al. (1994) found that protein-bound plasma T4 in mice changed only from 99-94% of total plasma T4 in wildtype to 99-89% of total plasma T4 in TTR null mice. The change amounted to only 0-05%, despite the complete absence in the blood of TTR, the main T4-binding plasma protein in mice!

**TTR gene expression and ontogeny**

The investigation of the effect of a null mutation on the function of a redundant component in a network system is not an appropriate approach for the characterization of the contribution of an individual component to the complex function of the system. Some insight into the function of such a component can be gained from studying its regulation and evolution. The expression of the TTR gene in rat brain shows a very interesting correlation with growth and development of the brain (Fung et al. 1988). Maximum expression of the TTR gene occurs a few days before birth and precedes maximum growth (Fig. 4). This is precisely the time when the maximum number of neuroblast replications is observed in the rat brain (Morreale de Escobar et al. 1983). In contrast, the levels of cystatin C mRNA and of β2-microglobulin mRNA increase at the same time as the weight of the brain. An increase in the level of transferrin mRNA occurs later than that of the weight of the brain. It appears that TTR gene expression in the rat brain is maximal during differentiation, which is then followed by bulk growth. Lack of thyroid hormone before birth leads to stunting of dendrite growth, decreased deposition of myelin, a reduction in the number of synapses and the death of granule cells (Dussault & Ruel 1990).
1987). The TTR gene starts to be expressed in the cells of the choroid plexus anlage at the time when the blood–brain barrier is beginning to form (Thomas et al. 1988).

**Evolution of the tissue pattern of TTR gene expression**

In the last 15 years, TTR synthesis and gene expression in the liver and the brain have been studied in a large number of species, measuring TTR mRNA in tissues and TTR in blood (for review see Schreiber & Richardson 1997).

Recently, the nucleotide sequences of whole genomes became available for various non-human species. They were searched for TTR gene-like sequences. Thus, open reading frames (ORFs) for TTR gene-like nucleotide sequences have been found in *Salmonella dublin*, *Escherichia coli* and other bacteria, in the yeast *Schizosaccharomyces pombe*, and in the nematode *Caenorhabditis elegans* (Prapunpoj et al. 2000b). It is an open, interesting question whether these ORFs are transcribed into mRNA and translated into proteins.

In a fish, the seabream (*Sparus aurata*), TTR mRNA has been detected by Northern analysis in liver and by RT-PCR in other tissues (Santos & Power 1996, 1999, Funkenstein 1999). Other fish species have not yet been studied.

In amphibians, TTR mRNA was observed in the liver during metamorphosis, namely in tadpoles of the frog *Rana catesbeiana* (Yamauchi et al. 1998), and of the African clawed toad *Xenopus laevis* (Prapunpoj et al. 2000b). No TTR mRNA was observed in tadpole brain and in any of the studied tissues from adult amphibians. Very high levels of the mRNA for a lipocalin-type protein (binding lipophilic compounds), instead of TTR mRNA, were found in the choroid plexus of the cane toad *Bufo marinus* (Achen et al. 1992) and the bullfrog (Yamauchi et al. 1998).

In reptiles, the TTR gene was strongly expressed in the choroid plexus of adult lizards (*Tiliqua rugosa* (Achen et al. 1993)), turtles (*Trachemys scripta* (Richardson et al. 1997)) and young crocodiles (*Crocodylus porosus* (Prapunpoj 2002)), but not at all in their livers. TTR was also absent from the blood of turtles (Richardson et al. 1997). Also monotremes do not show TTR synthesis in their liver (Richardson et al. 1994).

TTR gene expression was observed in the choroid plexus of all studied marsupials. The picture for the distribution of TTR gene expression in the liver of marsupial species is more complex. Marsupials can be grouped into two suborders, based on their dentition, the Polyprotodonta (‘many front teeths’), and the Diprotodonta (‘two front teeths’). Marsupials live in both America and Australia. All American marsupials are polyprotodont. Some Australian marsupial species are polyprotodont, others are diprotodonts. The TTR gene was found to be expressed in the liver in all of the 45 studied Australian diprotodont species (from the families Macropodidae, Petauridae, Phalangeridae, Vombatidae, Burramyidae, Acrobatidae, Phascolarctidae, Tarsipedidae and Potoroidae) (Richardson et al. 1994, Duan et al. 1995). The TTR gene was not expressed in the liver of adult individuals of any of the 17 studied Australian polyprotodont marsupial species (from the families Dasyuridae, Peramelidae and Thylacomyidae) (Richardson et al. 1994).

American marsupials are all polyprotodont. Some express TTR in the liver, others do not (Richardson et al. 1996). Phylogenetic trees have been constructed for marsupial evolution based on reproductive features or on serological resemblances (Reig et al. 1987). Species found at the tips of the branches of such a tree for the American
marsupials (Dromiciops, Caluromys, Didelphis, Marmosa and Monodelphis) were all found to express TTR in the liver, whereas species located along the branches, namely Philander, Chironectes and Metachirus, showed no TTR gene expression in the liver of adults (Richardson et al. 1996). It seems likely, therefore, that the common ancestor of the American marsupials was polyprotodont and did not express the TTR gene in the liver.

Marsupials evolved first in America. In Gondwanaland, they spread via Antarctica to Australia (Woodburne & Zinsmeister 1982). Australia separated from Antarctica about 38–43 million years ago (Talent 1984), and, since that time, has been progressively moving northwards at about 7–8 cm per year (Li & Powell 2001). The diprotodont marsupials evolved in Australia independently from the marsupials in South America. It seems most likely that their last common ancestor was polyprotodont and did not synthesize TTR in its liver. It follows that TTR gene expression in the liver probably evolved independently in the Australian diprotodont and in the later appearing of the American polyprotodont marsupials.

Birds and placental mammals (eutherians) all express the TTR gene in both the choroid plexus and the liver (for review see Schreiber & Richardson 1997).

A maximum parsimony analysis was performed with the amino acid sequences of known TTR genes (Prapunpoj et al. 2000a). Such an analysis shows a monophyletic tree for TTR (Fig. 5). The position of the appearance of TTR in hypothetical common ancestors is indicated: the first appearance of TTR gene expression in the liver is marked ‘AL’ (studied specimens were adults), the first appearance of TTR gene expression in choroid plexus is marked ‘CP’.

In amphibians, the TTR gene is expressed in the liver during the metamorphosis of tadpoles only and this is labeled ‘JL’. It is likely that the common ancestors of all placental mammals expressed the TTR gene in the liver since all extant eutherians exhibit this property.

Diprotodont marsupials are herbivores, polyprotodont marsupials are carnivores or omnivores. The intestines of herbivores are much larger than the intestines of carnivores (Hume 1982). The pools for lipid-soluble compounds are larger in the herbivore than in the carnivore intestinal tract. One may speculate that this could be related to the selection pressure favoring the appearance of an additional thyroid hormone-binding protein, namely TTR, in the bloodstream of the diprotodonts.

Birds and reptiles have a reptile-like common ancestor. However, TTR synthesis in the liver evolved only in birds. Birds are endothermic, reptiles are ectotherms. In general, the relative size of internal organs of endotherms is larger than that in ectotherms. Internal organs, in particular the liver, are relatively rich in lipid membranes. Again, it might be possible that the selection pressure leading to the evolution of TTR gene expression in the liver of endotherms may be linked to an increase in lipid-soluble pools and that TTR helps to counteract the disappearance of thyroid hormone from the bloodstream into tissues by permeation into cells and cell membranes.

The appearance of TTR synthesis in the choroid plexus is correlated with the enormous increase in relative size of the brain in vertebrate evolution. Reptiles are the first species showing traces of a cortex (Kent 1987). Since the blood–brain barrier restricts the access of the other thyroid hormone-binding plasma proteins to the brain (that is albumin and TBG, both made only in the liver), a thyroid
Table 1. Comparison of the amino acid sequences of the TTRs from 20 vertebrate species. The numbering system is based on the sequence of human TTR. The symbols -/afii9825, -/afii9826, -/afii9828 are used for the amino acid residues introduced to align sequences with that of human TTR. The symbol # indicates the position of the exon 1/exon 2 border; double underlining indicates the T4 binding site. From Prapunpoj et al. (2000a), with permission from The Society for Molecular Biology and Evolution.

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<td>K<strong>K</strong>K<strong>E</strong></td>
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<td>E<strong>E</strong>E<strong>L</strong></td>
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<td>Ostrich</td>
<td>K<strong>K</strong>K<strong>E</strong></td>
<td>W<strong>P</strong>P<strong>A</strong></td>
<td>E<strong>E</strong>E<strong>L</strong></td>
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<td>Bullfrog</td>
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<td>Sea Bream</td>
<td>K<strong>K</strong>K<strong>E</strong></td>
<td>W<strong>P</strong>P<strong>A</strong></td>
<td>E<strong>E</strong>E<strong>L</strong></td>
<td>R<strong>E</strong>F<strong>V</strong></td>
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Figure 6. Comparison of the amino acid sequences of the TTRs from 20 vertebrate species. The numbering system is based on the sequence of human TTR. The symbols α, β, γ are used for the amino acid residues introduced to align sequences with that of human TTR. The symbol # indicates the position of the exon 1/exon 2 border; double underlining indicates the T4 binding site. From Prapunpoj et al. (2000a), with permission from The Society for Molecular Biology and Evolution.
hormone-binding protein synthesized within the brain (such as TTR) would be of particular importance during evolution of larger brains.

Two streams of research have greatly advanced our knowledge of the TTR gene in recent years. The first type of research arose from the investigation of the clinical consequences of mutations of the TTR gene, the second stems from the wish for understanding the relationship between structure and evolution of TTR.

About 80 mutations of the human TTR gene are known. Most of these lead to amyloidopathies, that is the inappropriate, pathological deposition of TTR with altered structure in tissues as amyloid (for reviews see Schreiber & Richardson 1997, Connors et al. 2000, Damas & Saraiva 2000, Hamilton & Benson 2001). These mutations are evenly spread along the polypeptide chain (Eneqvist & Sauer-Ericksson 2001). The deposition of amyloid eventually leads to serious disease. Time course and site of deposition vary for the different mutant TTRs.

## Evolution of the structure of the TTR gene

Two streams of research have greatly advanced our knowledge of the TTR gene in recent years. The first type of research arose from the investigation of the clinical consequences of mutations of the TTR gene, the second stems from the wish for understanding the relationship between structure and evolution of TTR.

Figure 7 Nucleotides flanking the splice sites of intron 1 in TTR precursor mRNAs. The upper half of the Figure represents the sequences around the exon 1/intron 1 border. The lower half of the Figure shows the intron 1/exon 2 border. The splice sites are indicated by two-ended arrows. The consensus recognition sequences for splicing are given in bold above the position of the splice site. From Prapunpoj et al. (2000a), with permission from The Society for Molecular Biology and Evolution.

### Table 2 Dissociation constants for thyroid hormones and TTRs from ten vertebrate species. Crocodile and Xenopus laevis TTR were expressed in the recombinant yeast Pichia pastoris. Data from Chang et al. (1999), and Prapunpoj et al. (2000b, 2002)

<table>
<thead>
<tr>
<th>TTR Source</th>
<th>$K_d$ T3 (nM)</th>
<th>$K_d$ T4 (nM)</th>
<th>$K_d$ T3/Kd T4</th>
</tr>
</thead>
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<tr>
<td>Eutherians</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Humans</td>
<td>56·6</td>
<td>13·6</td>
<td>4·2</td>
</tr>
<tr>
<td>Sheep</td>
<td>63·5</td>
<td>11·3</td>
<td>3·2</td>
</tr>
<tr>
<td>Rats</td>
<td>67·2</td>
<td>8·0</td>
<td>8·4</td>
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<tr>
<td>Marsupials</td>
<td></td>
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<tr>
<td>Wombat</td>
<td>97·8</td>
<td>21·8</td>
<td>4·5</td>
</tr>
<tr>
<td>Possum</td>
<td>206·1</td>
<td>15·9</td>
<td>12·9</td>
</tr>
<tr>
<td>Wallaby</td>
<td>65·3</td>
<td>13·8</td>
<td>4·7</td>
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<tr>
<td>Birds</td>
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<tr>
<td>Emu</td>
<td>18·9</td>
<td>37·4</td>
<td>0·51</td>
</tr>
<tr>
<td>Chicken</td>
<td>12·3</td>
<td>28·8</td>
<td>0·43</td>
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<tr>
<td>Pigeon</td>
<td>16·1</td>
<td>25·3</td>
<td>0·64</td>
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<tr>
<td>Reptiles</td>
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<tr>
<td>Saltwater crocodile</td>
<td>7·56</td>
<td>36·73</td>
<td>0·21</td>
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<td>Amphibians</td>
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<tr>
<td>Xenopus laevis</td>
<td>248</td>
<td>508</td>
<td>0·49</td>
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</table>
The second stream of research is concerned with the evolution of the TTR gene. About two dozen TTR genes from fish, amphibians and reptiles to birds and mammals were cloned and sequenced. The pattern of the location of the mutations along the polypeptide chain (Fig. 6) is quite different from that in human TTR leading to amyloidosis. For most of the polypeptide chain the sequence of amino acids is well conserved, in particular in the central channel of the TTR tetramer, which harbors the thyroid hormone-binding site (for review see Schreiber & Richardson 1997). Most of the mutations of TTR during evolution occur in the first ten amino acids from the N-terminus. They are not randomly located. From amphibian to reptile and bird TTR to polyprotodont and diprotodont TTR, and finally mammalian TTR, the N-terminal section of TTR is shortened successively and becomes more hydrophilic. For maintaining maximum alignment, several amino acids have to be inserted between amino acid 3 and 4 for comparison with the human TTR sequence. In Fig. 6, they are labeled with Greek letters. A negative prefix indicates the direction of counting from the right to the left.

**The molecular mechanism of the evolution of the structure of the TTR gene**

The evolution of the structure of the 5’-terminal section of the TTR gene (corresponding to the N-terminal section of the TTR subunit) occurs in a sequence of small steps leading to shorter and more hydrophilic ends of the TTR subunits. Adaptation to the environment in small steps is a key point in Darwin’s postulates for evolution by natural selection. Small improvements of function have to be inherited from one generation to the next to give a selection advantage.

What could be the molecular mechanism underlying these changes? The site at which additional amino acids are inserted into the N-terminal section of human TTR to achieve optimum alignment with reptile/bird/amphibian TTRs (Fig. 6) is the border between exon 1 and exon 2 of...
TTR (Fig. 7). An analysis and comparison of genomic DNA and cDNA nucleotide sequences in this region shows that the sequence at the 3′-end of exon 1 does not change, whereas a stepwise shift of the splice recognition site appears to occur at the 5′-end of exon 2 (Aldred et al. 1997). Closer inspection of the sequences suggests that this shift might have occurred by a series of single base mutations converting codons for amino acids into splice recognition sites. For example, a change of the codon CAU, coding for histidine in marsupial/bird/reptile TTR in position-α, into the sequence CAG by a change in the third base of the codon, would produce the new splice recognition site seen in mammalian TTR.

Selection pressure involved in TTR evolution.
Relationship between N-terminal sections of TTR subunits and function of TTR

TTR is a tetramer with four identical subunits. The tetramer contains a central channel with the two binding sites for thyroid hormones (Blake et al. 1978). There is negative cooperativity between the two sites (Ferguson et al. 1975, Neumann et al. 2001). The best conserved regions of the polypeptide chain are the binding site and the amino acids lining the channel (Schreiber & Richardson 1997, Prapunpoj et al. 2000a,b). It is only relatively recently that data became available for the three-dimensional structure of the first 10 amino acids from the N-termini of human TTR. Residues 1–9, the so-called @ loop, form a semicircle on the surface of each TTR monomer (Hamilton et al. 1993). The comparative data on the evolution of this section, discussed above, suggest that the N-terminal region of the polypeptide chain of TTR has a function (on which selection can operate) and that there may be a directional change of this function (see below) during the evolution of vertebrates.

Recently, the binding affinities were measured for different vertebrate species (Chang et al. 1999, Prapunpoj 2000b, 2002). In birds and reptiles, T3 was more tightly bound by TTR than T4. The binding affinities of TTR changed during evolution and mammalian TTR is binding T4 more tightly than T3 (Table 2).

To investigate the contribution of the N-terminal section of TTR to the binding of T4 and T3, TTR cDNA was expressed in the recombinant yeast Pichia pastoris. Plasmids were constructed which coded for chimeric TTRs (N-terminal section and the main part of the TTR stemming from different species). The synthesized and secreted TTRs were purified from the incubation medium. Binding of T3 and T4 was quantitated by Scatchard analysis. The N-terminal section was found to modulate specificity and affinity of T4 and T3 binding by TTR (Prapunpoj et al. 2002).

The ‘environment’ in which TTR would have to adapt to changes during evolution is, of course, different in the liver and in the brain. The selection pressure operating during evolution could be related to the function of TTR in the brain or that in the liver and the rest of the body. The observation that both mammals and birds are endotherms, but that only mammalian TTR developed a new N-terminal section of TTR, would point to the evolution of the mammalian brain as providing the selection pressure for the shortening and becoming more hydrophilic of the N-terminal section of TTR. The changes of structure and function of the brain were far greater than those of other organs during the evolution of the vertebrates. It is an intriguing, unanswered question whether and when selection pressure during evolution might operate on $K_d$ and when on on-rate or off-rate constants.

Integration of the evolution of TTR and of the regulation of metabolism by thyroid hormones

T3 is more potent than T4 in regulating transcription by thyroid hormones. Tissue-specific deiodinases converting T4 into T3 can lead to tissue-specific regulation of metabolism by thyroid hormones (Schrodervanderelst et al. 1997, Kohrle 1999, 2000). Of particular importance is the brain-specific 5′-deiodinase (type II deiodinase) in mammals. The contribution of the local production of T3 from T4 to total intracellular T3 varies from site to site in the brain (van Doorn et al. 1985). It is highest in the cortex, with 65%, and lowest in the pons (35%) and the medulla oblongata (30%), i.e. regions further removed from the choroid plexus. The rat choroid plexus itself does not contain any deiodinase activity; despite the local abundance of T4, no T3 formation was detected in choroid plexus (Southwell et al. 1993). Type II deiodinases, similar in properties to the enzyme in mammalian brain, have been described in liver, kidney, skin and other tissues in fish (MacLatchy & Eales 1992), metamorphosing bullfrog tadpoles (Galtion & Hiebert 1988), and saltwater crocodiles (Shepherdley et al. 2002). No such deiodinase, or only minimal amounts, were found in brain. More data are required to decide whether brain-specific production and regulation of T3 evolved at about the same time as the synthesis in the choroid plexus of TTR with increasing affinity for T4.

Concluding remarks

Pools and compartments important for the distribution of T4 are depicted in Fig. 8. The transfer of T4 between pools is indicated by arrows. The differential equations describing the association and dissociation reactions are given below the figure. The cartoon is an attempt to integrate the relationships of the various pools as a first basis for understanding thyroid hormone homeostasis in the body. It is a gross oversimplification. The derivation of
the differential equations, for example, involves the assumption of homogeneous pools of T4 from which infinitely small amounts, dT4, randomly taken, disappear in an infinitely small amount of time, dt. In reality such pools do not exist. Mixing in the bloodstream is relatively fast (in the human the lung–ear time is 3–5 s, the arm–ear time 8–12 s (Krayenbuhl 1975)). However, there is no fast, overall mixing of the CSF in the ventricular system of the brain. The choroid plexus secretes the CSF. It flows through the ventricular system in a pipe-line type movement and finds access to the bloodstream, partly through sites near the cribiform plate, and from there into the interstitium of the nasal mucosa (main passage for water, ions and small molecules), and partly through the deep lymph system of the neck (main passage for large molecules, e.g. proteins) (Bradbury 1993). In most mammals, it takes about 200 min for the secretion of an amount of CSF replacing the total volume of CSF in the ventricles (Cserr 1971). For rats, longitudinal relaxation time-weighted magnetic resonance imaging after intraventricular injection of the T₁-relaxation reagent Gd-DTPA showed that the whole ventricular space is not a single compartment (Takamata et al. 2001). Probably, the flow of CSF through the ventricular system involves both laminar and turbulent flow, the proportions of which differ from region to region. The intensity of CSF mixing in immediate proximity of the choroid plexus epithelial cells will be strongly influenced by the beating of the microvilli. The apical microvilli of the epithelial cells of the choroid plexus possess a very large surface area. In the rat, it is 75 cm² (Keep & Jones 1990), that is in the same range as the total surface area of the capillaries of the blood–brain barrier (155 cm²).

The complexity and functional redundancy of the factors involved in maintaining thyroid hormone homeostasis restrict the value of null mutations of thyroid hormone-binding protein genes for gaining insight into the contribution of single individual components. Highly functionally redundant networks for maintaining homeostasis provide an enormous selection advantage in evolution. A lack of a change in phenotype is likely to indicate that a particular function is so important that a highly redundant system has evolved, guaranteeing survival when one of the components of the system fails.

Acknowledgements

The integrating interpretation of the evolution of TTR presented here is based on the data obtained with the help of many young, enthusiastic PhD students who worked with me during the last 17 years. Many of them stayed on for a few years as postdoctoral fellows. In particular, I am grateful to A R Aldred, L Chang, P Dickson, W Duan, W-P Fung, P Porntip, S J Richardson and B R Southwell. I also very much enjoyed the collaboration of M Achen (postdoctoral fellow) and T Pettersson and M Segal (sabbatical visitors).

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