Structural and functional properties of transthyretin

av

Anders Karlsson

Akademisk avhandling

som med vederbörligt tillstånd av rektorsämbetet vid Umeå Universitet för avläggande av filosofie doktorsexamen i molekylärbiologi framläggs till offentligt försvar i sal B120, byggnad 1D, NUS, fredagen den 15 februari 2008, kl. 09.00.

Avhandlingen kommer att försvaras på engelska.

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Umeå Center for Molecular Pathogenesis
Umeå University
Umeå 2008
Abstract
The hereditary transthyretin (TTR) amyloidoses are rare, and in severe cases, fatal disorders caused by mutations in the TTR gene. The clinical picture is diverse, involving neuropathies and myopathies, and mainly depends on the causative mutation and the sites and rates of amyloid deposition. The ultimate aim of the field of research presented in this thesis is to prevent TTR amyloid disease. To reach this ambitious goal, a thorough understanding of the normal as well as the pathological properties of the protein is essential. Here, comparisons between TTR from humans and other species may provide valuable information.

The three-dimensional structure of TTR from Gilthead sea bream (Sparus aurata) was determined at 1.75 Å resolution by X-ray crystallography, and was found to be structurally similar to human TTR. However, significant differences were observed in the area at and around β-strand D, an area believed to dissociate from the structure prior to amyloid formation, thereby allowing the β-strands A and B to participate in polymerization. During evolution, the preference of TTR for the thyroid hormones, 3,5,3'-triiodo-L-thyronine (T3) and 3,5,3',5'-tetraiodo-L-thyronine (T4), has shifted. While human TTR has higher affinity for T4, the opposite is true in lower vertebrates, e.g. fish and reptiles, where T3 is the main ligand. We have determined two separate structures of sea bream TTR in complex with T3 and T4, both at 1.9 Å resolution, as well as the complex of human TTR with T3. A significantly wider entrance and narrower thyroid hormone binding channel suggest a structural explanation to the differences in thyroid hormone preference between human and piscine TTR.

The Tyr114Cys substitution in TTR is associated with severe systemic amyloidosis. The mutation introduces a second cysteinyl group in the TTR monomer, and has been shown to inhibit the formation of fibril formation in vitro, promoting the formation of disulfide-bonded amorphous aggregates. To deduce the role of intermolecular disulfide bonds in fibril formation we characterized the TTR Cys10Ala/Tyr114Cys double mutant. Our results suggest that an intermolecular disulfide bond at position 114 enhances the exposure of Cys10, which promotes the formation of additional intermolecular disulfide-linked assemblies. Also, we were able to isolate a disulfide-linked dimeric form of this mutant that formed protofibrils in vitro, suggesting the architecture of TTR amyloid may be the result of different underlying structures rather than that of a highly stringent assembly.

We have also been able to successfully adapt a method of protein pre-heating to enable crystallization, thereby succeeding in a particularly problematic protein crystallization experiment. By heating the protein solution, we succeeded in separating several forms of protein micro-heterogeneities from the properly folded protein species, thereby allowing the growth of well diffracting crystals.

Keywords: Transthyretin, Amyloidosis, X-ray crystallography, Protein structure, Protein crystallization

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Umeå Center for Molecular Pathogenesis
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Umeå 2008
Gör man rätt så blir det rätt
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ABSTRACT

The hereditary transthyretin (TTR) amyloidoses are rare, and in severe cases, fatal disorders caused by mutations in the TTR gene. The clinical picture is diverse, involving neuropathies and myopathies, and mainly depends on the causative mutation and the sites and rates of amyloid deposition. The ultimate aim of the field of research presented in this thesis is to prevent TTR amyloid disease. To reach this ambitious goal, a thorough understanding of the normal as well as the pathological properties of the protein is essential. Here, comparisons between TTR from humans and other species may provide valuable information.

The three-dimensional structure of TTR from Gilthead sea bream (*Sparus aurata*) was determined at 1.75 Å resolution by X-ray crystallography, and was found to be structurally similar to human TTR. However, significant differences were observed in the area at and around $\beta$-strand D, an area believed to dissociate from the structure prior to amyloid formation, thereby allowing the $\beta$-strands A and B to participate in polymerization. During evolution, the preference of TTR for the thyroid hormones, $3,5,3'$-triiodo-L-thyronine (T$_3$) and $3,5,3',5'$-tetraiodo-L-thyronine (T$_4$), has shifted. While human TTR has higher affinity for T$_4$, the opposite is true in lower vertebrates, e.g. fish and reptiles, where T$_3$ is the main ligand. We have determined two separate structures of sea bream TTR in complex with T$_3$ and T$_4$, both at 1.9 Å resolution, as well as the complex of human TTR with T$_3$. A significantly wider entrance and narrower thyroid hormone binding channel suggest a structural explanation to the differences in thyroid hormone preference between human and piscine TTR.

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PUBLICATIONS

This thesis is based on the following papers, which will be referred to by roman numerals (Paper I-III).


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Reprinted with permission. Copyright 2005 American Chemical Society.


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Structure coordinates from this thesis are deposited at the RCSB protein data bank with the following identification codes: 1SN0, 1SN2, 1SN5, 1IIN, 2QEL.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A...</td>
<td>In vivo amyloidogenic form of a protein, e.g., ATTR for amyloidogenic transthyretin</td>
</tr>
<tr>
<td>Apo-A1</td>
<td>Apolipoprotein A-1</td>
</tr>
<tr>
<td>BME</td>
<td>β-mercaptoethanol, 2-mercaptoethanol</td>
</tr>
<tr>
<td>CAA</td>
<td>Cerebral amyloid angiopathy</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>FAC</td>
<td>Familial amyloidotic cardiomyopathy</td>
</tr>
<tr>
<td>FAP</td>
<td>Familial amyloidotic polyneuropathy</td>
</tr>
<tr>
<td>GAGs</td>
<td>Glycosaminoglycans</td>
</tr>
<tr>
<td>HBP</td>
<td>Halogen-binding pocket</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>OLT</td>
<td>Orthotopic liver transplantation</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>RBP</td>
<td>Retinol-binding protein</td>
</tr>
<tr>
<td>Rms(d)</td>
<td>Root mean square (deviation)</td>
</tr>
<tr>
<td>SAP</td>
<td>Serum amyloid component P</td>
</tr>
<tr>
<td>SSA</td>
<td>Senile systemic amyloidosis</td>
</tr>
<tr>
<td>T₃</td>
<td>3, 5, 3′-triiodo-L-thyronine</td>
</tr>
<tr>
<td>T₄</td>
<td>3, 5, 3′, 5′-tetraiodo-L-thyronine, thyroxine</td>
</tr>
<tr>
<td>TBPA</td>
<td>Thyroxine-binding prealbumin</td>
</tr>
<tr>
<td>THDPs</td>
<td>Thyroid hormone distributor proteins</td>
</tr>
<tr>
<td>TLP</td>
<td>Transthyretin-like protein</td>
</tr>
<tr>
<td>TRP</td>
<td>Transthyretin-related protein</td>
</tr>
<tr>
<td>TSH</td>
<td>Thyroid-stimulating hormone</td>
</tr>
<tr>
<td>TTR</td>
<td>Transthyretin</td>
</tr>
<tr>
<td>wtTTR</td>
<td>Human wild type transthyretin</td>
</tr>
</tbody>
</table>
PREFACE

The human body contains approximately 100,000 different proteins. They are the work horses of the cells and are intimately linked to virtually every chemical process of the organism.

The three dimensional structure of a protein is largely determined by the sequence of its amino acid residues. Perhaps in contradiction to what one might think, a protein structure is not a rigid entity but a highly dynamic system with a high degree of conformational freedom. The flexibility is an inherent feature of proteins and a prerequisite for their normal function, but is also a potential hazard. An abnormal increase of the flexibility results in a destabilised protein that is less suited to perform its function, and may have devastating consequences for the organism. Evolution has selected for proteins with a reasonable compromise between conformational flexibility and structural integrity. However, the balance is delicate, and may be shifted by mutations and post-translational modifications. Mutations result in changes of the primary sequence of the protein and may disturb the network of inter- and intramolecular interactions that are responsible for the structure and function. Sometimes this is beneficial to the organism, resulting in a more functional protein, but often it is detrimental, leading to an excessive stabilization or destabilization of the structure, eventually yielding a misfolded and malfunctioning protein. When the effects of these events are added, we may end up with a protein where the normal function is impaired to the extent where it is actually the cause of pathology. In the protein transthyretin, normally involved in distribution of thyroid hormones and retinol throughout the body, different mechanisms result in misfolding and pathological deposition of amyloid. In this thesis, several aspects of transthyretin is discussed in relation to such misfolding events, but also in respect of ligand binding and more methodological aspects related to the study of its structural properties.
INTRODUCTION

1. AMYLOID AND AMYLOIDOSIS

1.1. HISTORICAL OVERVIEW

One of the earliest published records of amyloid dates back to 1838, when Schleiden used the term to describe a macroscopic structure in plants that stained blue with iodine. The blue colour reaction was a well known feature of starch; hence the Greek word amylon (translated as starch) was transcribed to amyloid, for starchlike. In 1854, Virchow adapted the iodine staining techniques to human tissues, identifying areas in the nervous system that were stained with iodine. Based on this staining, he suggested that these areas consisted of starch. A few years after the publication of Virchow’s findings, Friedrich and Kekulé characterised amyloid of the liver and found no chemical evidence of starch being present and concluded that the deposits most likely consisted of protein. However, the use of amyloid as the term for these deposits was not changed (for a historical review, see reference (4)).

1.2. AMYLOIDOSIS

The current recommendations of The Nomenclature Committee of the International Society of Amyloidosis are that amyloid should be defined as an in vivo deposited material mainly consisting of protein. The deposits should have a fibrillar morphology observable by electron microscopy, a typical cross-β X-ray diffraction pattern, and stain with amyloid specific histological stains. In particular, the deposits should have a marked affinity for the dye Congo red, and display a green birefringence upon binding (5).

In table 1, the 26 structurally and functionally different proteins that have been found to form pathological amyloid deposits in humans are listed (5). In their native form, these proteins are stable and soluble, but for different reasons they can acquire an increased propensity for abnormal self-aggregation, ultimately resulting in the accumulation of amyloid in disorders such as Alzheimer’s disease and Type II diabetes.
<table>
<thead>
<tr>
<th>Amyloid protein</th>
<th>Precursor</th>
<th>Systemic (S) or localized, organ restricted (L)</th>
<th>Syndrome or involved tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL</td>
<td>Immunoglobulin light chain</td>
<td>S, L</td>
<td>Primary, Myeloma-associated</td>
</tr>
<tr>
<td>AH</td>
<td>Immunoglobulin heavy chain</td>
<td>S, L</td>
<td>Primary, Myeloma-associated</td>
</tr>
<tr>
<td>Aβ2M</td>
<td>β2-microglobulin</td>
<td>S</td>
<td>Hemodialysis-associated, Joints</td>
</tr>
<tr>
<td>ATTR</td>
<td>Transthyretin</td>
<td>L?</td>
<td>Familial, Senile systemic, Tenosynovium</td>
</tr>
<tr>
<td>AA</td>
<td>(Apo)serum AA</td>
<td>S</td>
<td>Secondary, reactive</td>
</tr>
<tr>
<td>AApoAI</td>
<td>Apolipoprotein AI</td>
<td>S</td>
<td>Familial</td>
</tr>
<tr>
<td>AApoAII</td>
<td>Apolipoprotein AII</td>
<td>S</td>
<td>Familial</td>
</tr>
<tr>
<td>AApoAIV</td>
<td>Apolipoprotein AIV</td>
<td>S</td>
<td>Sporadic, associated with aging</td>
</tr>
<tr>
<td>AGel</td>
<td>Gelsolin</td>
<td>S</td>
<td>Familial (Finnish)</td>
</tr>
<tr>
<td>ALys</td>
<td>Lysozyme</td>
<td>S</td>
<td>Familial</td>
</tr>
<tr>
<td>AFib</td>
<td>Fibrinogen a-chain</td>
<td>S</td>
<td>Familial</td>
</tr>
<tr>
<td>ACys</td>
<td>Cystatin C</td>
<td>S</td>
<td>Familial</td>
</tr>
<tr>
<td>ABri</td>
<td>ABriPP</td>
<td>S</td>
<td>Familial dementia, British, British</td>
</tr>
<tr>
<td>ADan*</td>
<td>ADanPP</td>
<td>L</td>
<td>Familial dementia, Danish</td>
</tr>
<tr>
<td>Aβ</td>
<td>Aβ protein precursor (AβPP)</td>
<td>L</td>
<td>Alzheimer’s disease, aging</td>
</tr>
<tr>
<td>APRP</td>
<td>Prion protein</td>
<td>L</td>
<td>Spongiform encephalopathies</td>
</tr>
<tr>
<td>ACaI</td>
<td>(Pro)calcitonin</td>
<td>L</td>
<td>C-cell thyroid tumors</td>
</tr>
<tr>
<td>AIAAPP</td>
<td>Islet amyloid polypeptide**</td>
<td>L</td>
<td>Islets of Langerhans, Insulinomas</td>
</tr>
<tr>
<td>AANF</td>
<td>Atrial natriuretic factor</td>
<td>L</td>
<td>Cardiac atria</td>
</tr>
<tr>
<td>APro</td>
<td>Prolactin</td>
<td>L</td>
<td>Aging pituitary, Prolactinomas</td>
</tr>
<tr>
<td>AIns</td>
<td>Insulin</td>
<td>L</td>
<td>Iatrogenic</td>
</tr>
<tr>
<td>AMed</td>
<td>Lactadherin</td>
<td>L</td>
<td>Senile aortic, arterial media</td>
</tr>
<tr>
<td>AKer</td>
<td>Kerato-epithelin</td>
<td>L</td>
<td>Cornea, familial</td>
</tr>
<tr>
<td>ALac</td>
<td>Lactoferrin</td>
<td>L</td>
<td>Cornea</td>
</tr>
<tr>
<td>AOaap</td>
<td>Odontogenic ameloblast-associated protein</td>
<td>L</td>
<td>Odontogenic tumors</td>
</tr>
<tr>
<td>ASEmI</td>
<td>Semenogelin I</td>
<td>L</td>
<td>Vesicula seminalis</td>
</tr>
<tr>
<td>ATau</td>
<td>Tau</td>
<td>L</td>
<td>Alzheimer’s disease, fronto-temporal dementia, aging, other cerebral conditions</td>
</tr>
</tbody>
</table>

*ADan comes from the same gene as ABri; **Also called ‘amylin’.
Previous recommendations on the nomenclature of amyloid and amyloidosis have defined amyloid as an extracellular protein assembly, but with an increasing amount of evidence for intracellular fibril formation in disorders such as Huntington’s and Parkinson’s disease, the recommended definition of amyloid now also encompasses such fibrils (table 2).

Table 2. Intracellular inclusions with known biochemical composition with or without amyloid properties, adapted from Westermark et al. (5).

<table>
<thead>
<tr>
<th>Inclusion name</th>
<th>Site</th>
<th>Protein nature</th>
<th>Examples of associated disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lewy bodies</td>
<td>Neurons</td>
<td>Intracytoplasmic a-synuclein*</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>Huntington</td>
<td>Neurons</td>
<td>Intranuclear poly-Q expanded huntingtin</td>
<td>Huntington’s disease</td>
</tr>
<tr>
<td>bodies</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hirano bodies</td>
<td>Neurons</td>
<td>Actin</td>
<td>Neurodegenerative disorders</td>
</tr>
<tr>
<td>Not specified</td>
<td>Neurons</td>
<td>Neuroserpin</td>
<td>Forms of familial presenile dementia</td>
</tr>
<tr>
<td>Not specified</td>
<td>Neurons, many different cells</td>
<td>Ferritin</td>
<td>Form of familial neurodegenerative disorder</td>
</tr>
</tbody>
</table>

In several forms of amyloidosis and amyloid associated disorders, the mere bulk of the deposited protein is enough to disrupt normal organ function, but studies have also shown a correlation between cell-line directed toxicity and the presence of pre-fibrillar aggregates. This suggests a more complex, multi factor dependent pathogenesis in amyloid disease (6,7).
1.3. COMMON PROPERTIES OF AMYLOID

Being a low energy folding state structure, amyloid shows an amazing chemical and physical stability. Regardless of the main constituent protein and the plethora of clinical syndromes associated with amyloid, the morphological and chemical characteristics of the deposits are very similar.

1.3.1. FIBRILLAR

The physical appearance of amyloid is fibrillar. Typical *ex vivo* fibrils are straight and un-branched. They are of indeterminate length and have a diameter of 70 Å to 140 Å, and appear to consist of several protofilaments wound around each other (8,9). The fibrils were early discovered to be highly ordered. X-ray fiber diffraction studies show meridional and equatorial reflections at 4.7-4.8 Å and 10 Å, respectively. These numbers roughly correspond to the distances normally observed between β-strands and β-sheet in protein structures, and suggest a common β-strand core of amyloid (10-12).

![Figure 1. The cross-β model of amyloid](image)
1.3.2. TINCTORIAL PROPERTIES

The internal order of the fibrils gives them tinctorial properties that are useful in the clinic as well as in the laboratory. The azo-dye Congo red is considered the gold standard for clinical diagnosis of amyloid. It is typically used in histological staining of amyloid, where it shows a typical red-green dichroism under polarized light when bound to amyloid (originally published by Divry & Florkin, see reference (13)). Thioflavin T is primarily used in pre-clinical research as an \textit{in vitro} staining and detection method. It exhibits a fluorescence spectral red-shift when bound to amyloid (14) which can be conveniently measured by standard fluorimetric methods.

1.3.3. ASSOCIATED PROTEINS

In regard of protein composition, amyloid is never homogenous. Different sulphated glycosaminoglycans (GAGs) are always present in or associated with the fibrils, and the pentraxin serum amyloid component P (SAP) has been shown to bind all forms of amyloid fibrils, protecting them from proteolytic degradation (15). This generality of SAP binding has been elegantly utilized in a clinical amyloid imaging and quantification technique, where injection of radiolabelled SAP followed by whole body scintigraphy provide a good measure of the amyloid burden of a patient (16,17).
1.4. PHYSIOLOGICAL AMYLOID

The term amyloid is intimately linked to severe disorders, and the common notion in the scientific community has been that amyloid is per definition bad. However, an increasing amount of evidence suggests that the amyloid formed by some proteins is not a fluke by nature, but an evolutionary conserved, low-energy quaternary structure. Several proteins have been found to adopt the amyloid form in a physiological manner; being inherently amyloidogenic but not causing amyloid disease (18). Such amyloid has been shown to play important roles in a wide variety of processes in different organisms where the physical strength and chemical resilience of the fibrils is an advantage rather than a liability (see table 3 for a more comprehensive listing). This new field of physiological amyloid is interesting by itself, but may also prove important in developing novel amyloidosis therapies, since understanding the normal physiological functions and functional aspects of amyloid will most likely contain important lessons of the events of the pathogenesis of the amyloidoses.

**Table 3.** Examples of functional amyloid from different organisms. Adapted from Fowler et al. (18).

<table>
<thead>
<tr>
<th>Species</th>
<th>Protein</th>
<th>Function</th>
<th>Experimental evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em>, <em>Salmonella spp.</em></td>
<td>Curli</td>
<td>Biofilm formation, host invasion</td>
<td>EM, Congo red, ThioT, CD</td>
</tr>
<tr>
<td><em>Streptomyces coelicolor</em></td>
<td>Chaplins</td>
<td>Modulation of water surface tension</td>
<td>CD, ThioT, EM</td>
</tr>
<tr>
<td><em>Podospora anserine</em></td>
<td>HET-s</td>
<td>Regulation of heterokaryon formation</td>
<td>EM, CD, FTIR, Congo red, NMR</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>URE2p</td>
<td>Regulation of nitrogen catabolism</td>
<td>Electron diffraction, X-ray, Congo red, EM, ThioT</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>Sup35p</td>
<td>Regulation of stop-codon read-through</td>
<td>EM, Congo red, CD, X-ray</td>
</tr>
<tr>
<td>Most fungi</td>
<td>Hydrophobins</td>
<td>Fungal coat formation, modulation of adhesion and surface tension</td>
<td>AFM, CD, FTIR, ThioT, NMR, Congo red, X-ray</td>
</tr>
<tr>
<td><em>Insects and fish</em></td>
<td>Chorion proteins</td>
<td>Structural and protective functions in the eggshell</td>
<td>Congo red, X-ray, EM, FTIR, CD</td>
</tr>
<tr>
<td><em>Nephila clavipes</em></td>
<td>Spidroins</td>
<td>Structural (i.e. spider silk)</td>
<td>EM, CD</td>
</tr>
<tr>
<td><em>Homo sapiens</em></td>
<td>Pmel17</td>
<td>Scaffolding and sequestration of toxic intermediates during melanin synthesis</td>
<td>X-ray, Congo red, EM, ThioT</td>
</tr>
</tbody>
</table>
2. TRANSTHYRETIN

Of the different forms of hereditary amyloidosis, the most common ones are caused by transthyretin (TTR), affecting about 10,000 people globally (19). The severity and prognoses of the different medical conditions caused by these amyloidoses span from being mostly asymptomatic and non-lethal to being severely debilitating and fatal (further discussed in section 2.5).

Transthyretin was discovered as a protein with a higher electrophoretic mobility than albumin. Consequently, it was named prealbumin. An understanding of its function was first obtained by Sydney Ingbar, who observed a strong binding of thyroid hormones to prealbumin. He coined the name thyroxine-binding prealbumin (TBPA) to reflect this property (20). This nomenclature was soon recognized as a source of confusion, since it also could be read as a pre-form of albumin. The name transthyretin, designed to reflect the dual transport function of thyroid hormones and retinol-binding protein (RBP, further discussed in section 2.2) was therefore suggested (21), and has since been the most recognized name.

2.1. GENOMIC ORGANISATION AND EXPRESSION

Transthyretin (TTR) is a 55 kDa homotetrameric protein consisting of four identical monomers, each comprising 127 amino acid residues (22,23). It is encoded by a single copy gene which spans approximately 7 kb with four exons and three introns (24). Exon 1 codes for a 20 residue signal peptide and the first three residues of the mature protein. Exon 2 codes for residues 4-47, exon 3 for residues 48-92, and exon 4 for residue 93-127. The gene is located at chromosome 18q11.2–q12.1 (25) in a single copy (26,27).

TTR is present in serum and cerebrospinal fluid. Serum TTR is mainly expressed in the liver, and has a normal concentration of 0.2-0.4 mg ml⁻¹ and a half-life of approximately 2 days. In the CNS, the expression of TTR is highest in the choroid plexus, reaching a cerebrospinal fluid concentration of 0.02-0.04 mg ml⁻¹ (28), but it also occurs in the retinal and ciliary pigment epithelia of the eye (29,30).

The plasma concentration of TTR is negatively correlated to the acute response of the liver and has been widely accepted as a clinical biomarker for malnutrition (31,32). However, arguments were recently raised that this use of TTR should be discouraged, mainly because the high natural variability of the plasma concentration, but also due to the fact that in addition to the nutritional intake sensitivity, TTR concentration is also affected by too many disease processes to be a reliable marker of the nutritional status (33).
2.2. FUNCTION

The most widely recognised roles of TTR are as a thyroid hormone transporter and as a binding partner in the holoRBP-TTR complex. Maintenance functions in the nervous system have been suggested, but yet no conclusive evidence for such function exists.

2.2.1. THYROID HORMONE TRANSPORT

The thyroid hormones T₄ (3,5,3',5'-tetraiodo-L-thyronine or thyroxine) and T₃ (3,5,3'-triiodo-L-thyronine) are extremely biologically important and have been identified in all groups of vertebrates (34). Primarily, they act on the transcription level in virtually every cell in the body, where they mediate a wide array of regulatory actions. In humans, the bulk of the thyroid hormones is transported through the circulation in the form of T₄, which has traditionally been viewed as an inactive pro-hormone. However, it was recently shown to interact with cell surface receptors (35,36). Compared to T₄, T₃ exhibits a stronger interaction with the nuclear thyroid hormone receptors and is more biologically active. It is formed by specific deiodination of T₄ by intracellular deiodinases in the target cells.

The synthesis of the hormones occurs on tyrosines of the protein thyroglobulin, in a complex reaction that involves cellular uptake and oxidation of iodide and the subsequent conjugation of iodine onto the tyrosines. After synthesis, the iodothyronated thyroglobulins are stored in the colloid. Release of the free hormones is triggered by pituitary thyroid-stimulating hormone (TSH) and the subsequent secretion is a result of hydrolysis of thyroglobulin (35). The secreted iodothyronines are virtually insoluble in water and would consequently be integrated into the plasma membranes of the neighbouring cells unless the thyroid hormone distributing proteins (THDPs) were present in the blood. Of these, the thyroxine binding globulin (TBG) has the highest affinity for T₄ (Kₐ=2.5·10⁹ M⁻¹) and binds approximately 75% of the circulating thyroid hormones (37). Transthyretin has a lower affinity (Kₐ(T₄)=3.5·10⁷ M⁻¹) and binds 15-20% (38), while albumin, having the lowest affinity (Kₐ(T₄)=5.0·10⁵ M⁻¹), binds 5-10% of the available hormones.
Transthyretin has two sites for hormone binding (Figure 2b), but due to negative cooperativity, only one is occupied at physiological concentrations (39). However, by adding a large molar excess of hormone in vitro, it is possible to fully saturate both hormone binding sites (40). In the CNS, TTR is the only identified thyroid hormone carrier.

2.2.2. TRANSPORT OF RETINOL-BINDING PROTEIN

Retinol is a fat soluble metabolite of vitamin A. It is secreted from the liver bound to the 21 kDa lipocalin retinol-binding protein (RBP). RBP is synthesized and secreted primarily by hepatocytes, and is the only specific transport protein for retinol in the circulation. By binding retinol, the structure of RBP is changed. It adopts a higher affinity for TTR, which promotes the formation of the holoRBP-TTR-complex (41). The complex formation rescues the small RBP molecule from glomerular filtration in the kidneys, and is part of the major pathway for distribution of retinol to the target tissues throughout the organism (42,43). The structure of the complex is further discussed in section 2.3.1.

2.2.3. CRYPTIC PROTEASE ACTIVITY

1-2% of human plasma TTR is located in the high density lipoprotein (HDL) particles in plasma (44). The major protein component of the HDLs is the apolipoprotein-A1 (Apo-A1). It is involved in the cellular efflux of cholesterol, and also participates in the formation, metabolism and catabolism of HDL cholesterol esters. Rare forms of late onset familial amyloidosis caused by Apo-A1 have been described (44-46), and it was suggested that TTR may contribute to the course of the disease by proteolytic cleavage of Apo-A1 at residue Phe225. Recent data supporting this hypothesis show
an increased amyloidogenicity for Apo-A1 cleaved by TTR, and also an impaired ability to promote cholesterol efflux (47). The manner in which TTR cleaves Apo-A1 is unclear, but a study by Liz et al. suggests that TTR is a serine protease with a chymotrypsin-like activity (48). However, the known normal function does not fit with the observed proteolytic activity. Also, none of the canonical protease motifs of the known classes of proteases are present in the TTR structure.

2.2.4. ROLES IN THE NERVOUS SYSTEM

The apparent lack of a functional connection between the transport protein and the nervous system has intrigued scientists for decades. However, recent findings in mouse model systems suggest roles for TTR in the maintenance and function of the nervous system.

Recent data provided by Sousa and co-workers suggest a correlation between the absence of TTR and an acceleration of the cognitive decline normally associated with ageing. At 5 months of age they observed an impaired spatial memory function in TTR -/- mice compared to wild type mice, but at 18 months, this difference was abolished (49).

Brouilette and Quiron also observed the cognitive deficiencies of TTR null mice. In their study, the deficiencies were reversible by the administration of retinoic acid (RA), the active metabolite of retinol. The authors suggest that the loss of TTR and the consequential disruption of the retinol transport pathway is the main cause of the observed learning deficiencies. Normally, retinol is transported through plasma by the holoRBP-TTR complex and is metabolised in the cells to RA. By direct administration of RA, which is mainly transported by serum albumin, the TTR pathway is bypassed and the transcription regulatory functions are restored (50).

Fleming and co-workers demonstrated a role of TTR in sensorimotor function, and also as an enhancer of nerve regeneration. The authors suggest that this is a consequence of direct interactions between the neurons and TTR, rather than a ligand mediated effect (51).
Transthyretin is a homotetrameric protein shaped as a dimer of dimers. Each monomer consists of eight β-strands, usually denoted A-H, and a short α-helix. The β-strands are organised into two four-stranded sheets, comprising strands D-A-G-H and C-B-E-F, respectively, in the form of a β-barrel like structure. The helix is located after the E-strand (see figure 3).

The dimer is formed mainly through antiparallel β-strand interactions between the H-strands, resulting in a continuous eight stranded β-sheet comprising strands D-A-G-H and H'-G'-A'-D'. The two F-strands from each monomer also interact, but predominantly through side chains and interconnecting water molecules.

The tetramer is shaped by interdimeric hydrophobic contacts between the AB- and the GH-loops, resulting in the formation of a central core that is divided into two hormone binding channels. Each channel has three symmetrically related regions with high affinity for halogens. These so-called halogen-binding pockets (HBPs) govern the binding of the thyroid hormones as well as other ligands, and for convenience they are usually designated HBP1-HBP1*, HBP2-HBP2* and HBP3-HBP3*, where * indicates the corresponding pocket in the symmetry related dimer (3). HBP1 is found at the distal part of the hydrophobic channel, close to the exterior surface of the protein, and is formed by the side chains of residues Met13, Lys15 and Thr106. HBP2, in the middle of the channel, is made up by the side chains of Lys15, Leu17, Ala109, Leu110, and the hydrophilic main chain carbonyl groups of Lys15, Ala108 and Ala109. HBP3 is situated closest to the centre of the tetramer, where it is defined by the side chains of Ala108, Leu110, Ser117 and Thr119, and the main chain carbonyl and amino groups of Ala108, Ala109, Leu110 and Thr118 (see figures 3c and 3d).

Over 80 of the 97 structures of TTR deposited in the Protein Data Bank at the time of writing (2008-01-12) were solved by X-ray crystallography. Most of these describe the human wild type and different mutant proteins, with and without ligands, but there are also some structures of TTR from other organisms. With a few exceptions, all structures of human TTR since the pioneering work of professor Colin Blake and co-workers in 1978 have been solved in the orthorhombic space group P21212 with a dimer in the asymmetric unit (52). Since functional transthyretin is homotetrameric with an internal 222 symmetry, the dimer in P21221 has to be rotated through a 2-fold axis to generate the functional tetramer. As discussed further below, this complicates the determination and interpretation of structures containing asymmetric ligands such as the thyroid hormones.
Figure 3. The three-dimensional structure of transthyretin. 

a) The monomer arrangement of the dimer (pdb code: 1F41 (2)). 
b) The tetramer shown with bound thyroxine (pdb code: 2ROX (3)). 
c) The main residues involved in the halogen binding of the hormone binding channels. Residues involved are colored coded as follows: HBP1, red; HBP1 and HBP2, coral; HBP2, yellow; HBP2 and HBP3, blue; HBP3, purple. 
d) as c) with thyroxine shown.
2.3.1. THE TTR-RBP COMPLEX

RBP is a mainly beta protein with one $\alpha$-helix and nine $\beta$-strands arranged in a $\beta$-barrel architecture. The hydrophobic part of retinol is bound in the central channel of the barrel, only exposing the terminal hydroxyl group to the solvent (53). The structure of the holoRBP-TTR complex was first solved as a chimera, comprising human TTR and chicken RBP (54). Later, the human holoRBP-TTR was crystallized and structurally determined in the complex. This structure provided new information regarding the mode of association, and also regarding the importance of the C-terminus of RBP. In chicken RBP, the C-terminal residues are disordered and not visible in the structure. In contrast, the all-human complex displays the C-terminus nestled in the binding interface between the proteins, where it increases the total buried surface with 40% compared to the chimeric complex (1). In plasma, tetrameric TTR and holoRBP form a stoichiometric 1:1 complex. This reflects the differences in plasma concentration rather than the binding capacities, since in vitro, a molar excess of RBP yields a complex with a 1:2 relationship (1). The interactions between human TTR and RBP are mainly hydrophobic, and involve the $\alpha$-helical region of TTR (residues 80-86) as well as the loop regions (residues 20-21, 81-84, 96-103, 114). In RBP, the two loop regions connecting strands 5 and 6 (residues 63-67), and 7 and 8 (residues 89-99), respectively, and the C-terminus of RBP (1,55) are the main interaction partners in forming the complex.

![Figure 4. The human holoRBP-TTR complex, pdb code: 1QAB (1).](image-url)
2.4. EVOLUTION OF TRANSTHYRETIN

Transthyretin has been identified in more than 20 vertebrate species, including eutherians, marsupials, amphibia, reptiles, birds and fish (56). Current data suggest that from an evolutionary standpoint, the earliest vertebrates with functional TTR are the lampreys (superfamily Euteleostei, family Agnatha), having a sequence identity of 36-47% compared to humans (57).

The primary structure of TTR from most vertebrates is highly conserved, especially in the regions involved in ligand binding. Nevertheless, the affinity for the different thyroid hormones has changed during evolution; eutherian and marsupial species have a higher affinity for T₄, while fish, amphibians, reptiles and birds show a preference for binding T₃ (for review see (56)).

The affinity of T₄ and T₃ has co-evolved with the length and hydrophobicity of the N-terminal region of TTR, going from longer and more hydrophobic to shorter and more hydrophilic. The main region affected by the evolutionary substitutions was identified as an incremental shift in the intron 1/exon 2 splice site, resulting in a shortening of exon 2, which has yielded a shorter and less hydrophobic N-terminus (58,59).

Pursuing this line of research, using different human/crocodile N-terminal chimeric TTRs as well as N-terminal truncated crocodile TTR, it was recently suggested that primarily the T₄ affinity is determined by the N-terminal residues, while T₃ binding is mainly determined by the hormone binding channel core region (60). Unfortunately, the N-terminal region is very mobile, and consequently it is usually not visible in the structures of TTR solved by X-ray crystallography.

2.4.1. TRANSTHYRETIN-RELATED PROTEINS – TTR ANCESTORS?

A family of TTR related proteins (TRPs) was recently identified in more than 80 different species. The DNA sequence analysis of this family suggests that it may be the founder gene of TTR, and that TTR in vertebrates is the result of an early gene duplication event. Structurally, the TRPs form tetramers similar to those formed by TTR, but lack the thyroid hormone affinity (61,62). The actual biological roles of the TRPs are not fully determined. However, a role in the metabolism of uric acid has been suggested, mainly based on observations of some TRPs having an uric acid hydrolase activity, (63,64).
2.5. TRANSTHYRETIN IN DISEASE

TTR is implicated in hereditary and idiopathic amyloidoses involving mostly mutant and wild-type protein, respectively (for recent reviews see (65,66). Considering the remarkable thermal and chemical stability of TTR (67,68), its apparently inherent propensity for aggregation is quite unexpected.

2.5.1. IDIOPATHIC TTR AMYLOIDOSIS

Wild type TTR is implicated in the most common systemic amyloidosis, previously called senile systemic amyloidosis (SSA) (69). This syndrome is strongly correlated with ageing, and affects approximately 25% of people over 80 years of age (70). It is often asymptomatic, with widespread low-level amyloid deposition in various organs. However, in unfortunate individuals, predominantly men, extensive myocardial amyloid infiltration occurs and eventually results in cardiomegaly and heart failure (71).

2.5.2. HEREDITARY TTR AMYLOIDOSIS

Hereditary systemic transthyretin amyloidosis, previously called familial amyloidotic polyneuropathy (FAP) and familial amyloidotic cardiomyopathy (FAC), affects about 10,000 people globally, making it the most common hereditary amyloidosis (19). The disorder is caused by any of about 100 identified substitution mutations in the TTR gene and exhibits an autosomal dominant inheritance with a very varied penetrance and severity of the symptoms.

The most common mutation leading to familial systemic TTR amyloidosis is the substitution from valine to methionine at residue 30. This syndrome was first described in 1952 as an unusual form of hereditary amyloidosis with sensorimotor peripheral polyneuropathy and autonomic dysfunction (72). TTR was identified in the amyloid deposits in 1978 (73), and the mutation (Val30Met) was discovered in 1983 (74,75). There is a wide geographic distribution of this mutation, including Japan (76), Portugal, the Americas (77) and Sweden (78). The age of onset varies between 20 to 70 years. The initial symptoms usually involve sensory peripheral neuropathy of the lower limbs, primarily affecting pain and temperature sensation. Severe neuropathy of the autonomic nervous system early in the disease is not uncommon, leading to problems with the gastrointestinal system, sexual impotence, and orthostatic hypotension (the clinical features of TTR amyloidosis are reviewed in ref (65)). Multigenic and environmental modulating factors have been proposed to contribute to the course of the disease, and may also explain the inconsistency in penetrance and severity of symptoms observed even between genetically identical individuals (79).
2.5.3. THE THERMODYNAMIC STABILITY AND DISSOCIATION KINETICS DETERMINES THE AMYLOIDOGENICITY OF TTR

With a dissociation temperature in the region of 100°C (22,68,80) and a urea denaturation midpoint concentration of 3.4 M (81), the stability of wild type TTR is remarkable (22). However, it exhibits an inherent propensity for dissociation and aggregation.

Mutations that destabilise the native fold of the protein are known predisposing factors for amyloid disease (82). This is true also for TTR, where destabilisation of the native fold of the protein is a prerequisite for amyloid formation. The events leading from the native protein to amyloid have been extensively studied, mainly in vitro, but the aggregation pathway of amyloid formation is still unclear.

One of the proposed models involves an initial, rate limiting tetramer dissociation step into monomers and dimers that precludes the actual amyloid formation (83,84). Depending on the thermodynamic stability of the protein, the monomers either reassemble into native tetramers or become further denatured, adopting a non-native, amyloidogenic fold. The equilibrium between the two pathways is stable until the amount of aggregated TTR becomes too large. At this point, amyloid is rapidly formed in a downhill, non-nucleation dependent process (85,86).

Recent observations are in disagreement with this model, showing in vitro protofibril assembly without the need for tetramer dissociation and a clear nucleation dependence fibril formation (87). It should also be noted that native-like dimers have been shown to be fibril formation competent, suggesting that complete dissociation into monomers is not a requirement for fibril formation of TTR (88).

The dissociation kinetics and the thermodynamic stability of TTR are determined by the primary sequence of the protein and are not directly coupled, suggesting an explanation for the observed differences in the penetrance and pathology of the disease related TTR mutants. The most severe amyloidosis is observed in patients with the Leu55Pro TTR, with an early onset and a penetrance of 100%. The aggressive course of the disease in these patients is explained by the thermodynamic and kinetic destabilisation of the protein resulting from the mutation. In comparison, the amyloidosis caused by the Val30Met mutation exhibits a low penetrance and higher age of onset (89). The main reason for this difference is believed to be due to the relatively slow dissociation kinetics of the Val30Met mutant. Stabilising mutations also exist in TTR: the Thr119Met mutation provides thermodynamic and kinetic trans-suppression of the amyloidogenicity of Val30Met/Thr119Met TTR, actually preventing amyloid formation (90,91). The nature of the amyloidogenic intermediate is not clearly defined. Several studies suggest that it consists of misfolded free monomers (92-94). However, data from NMR studies of the H/D exchange pattern of fibrils are consistent with modified dimers as the constituting units (95). In paper II, we showed that a Cys114 disulfide linked form of the TTR substitution mutant...
Cys10Ala/Tyr114Cys is compatible with fibril formation (96), supporting these findings. Serag et al. showed that native-like disulfide linked dimers can form protofibrillar structures \textit{in vitro}. Amyloidogenic intermediates in the form of tetramers were suggested by Eneqvist et al. This model was based on the structure of the highly aggregation prone TTR mutant Gly53Ser/Glu54Asp/Leu55Ser, which showed major conformational differences compared to the wild-type structure, and packing interactions in the crystal were reminiscent of fibrillar structures (97). The tetrameric intermediate was also proposed by Ferrao-Gonzales et al., who observed that protein compression induced a non-native tetrameric molecule that exhibited an increased propensity for amyloid formation (98).

\subsection*{2.5.4. THERAPY FOR TTR AMYLOIDOSES - TETRAMER STABILISATION AND LIVER TRANSPLANTATION}

The most successful treatment for hereditary systemic TTR amyloidosis is orthotopic liver transplantation (OLT). The rationale behind the method is that since most of the TTR in circulation is produced by the liver, replacing the liver removes the source of faulty TTR, i.e. a crude form of gene therapy. Since 1990, more than 1200 patients have been transplanted (99-103), and follow-up studies indicate that those carrying the Val30Met mutation benefit most from the OLT, while those with other forms of TTR amyloidosis have lower 5 year survival expectancy, and often show a progressive deposition of wild-type TTR amyloid, especially in the heart (101).

Since tetramer dissociation is the rate limiting step in fibril formation, stabilisation of the tetrameric TTR is being investigated as a potentially therapeutic approach for TTR amyloidosis (104,105). Non-steroidal anti-inflammatory drugs (NSAIDs) such as diflunisal and flufenamic acid have proven to be good tetrameric stabilisers \textit{in vitro} (106) and \textit{in vivo} (107,108) However, NSAID medication is still far from being a curative treatment, and long term studies on amyloidosis patients have not been undertaken. Current efforts aim at further modifying these drugs to increase the pharmacological properties (109,110), as well as developing novel drugs. In an ongoing collaboration with Prof. Fredrik Almqvist at the dept. of Chemistry, Umeå University, our group is pursuing small molecule inhibitor screening aimed at reducing the toxicity of the amyloidogenic intermediates (Lindhagen-Persson et al, unpublished data).
AIMS

The overall aim of this thesis is to forward the knowledge of the native properties and pathological mechanisms of transthyretin. More specifically, the aims are:

To reveal the structural determinants responsible for organism specific differences in binding of T₃ and T₄. In particular, we have aimed at characterizing the apo and thyroid hormone complex structures of Gilthead sea bream (Sparus aurata) TTR by X-ray crystallography. Piscine TTR was chosen, since at the time of the experiments, it was identified as the most evolutionary distant TTR compared to the human protein.

To determine the role of Cys10 in the in vitro fibril formation process of the disease related ATTR Tyr114Cys, and to investigate the implications for in vivo amyloid formation. This is of particular medical interest, since Cys10 modifications have been implied to increase the risk for TTR amyloid disease. Different modifications are commonly observed in ex vivo TTR, and have been shown to affect the stability of the protein in vitro.

During the course of my PhD studies, I have also been trying to reproduce a crystallizable form of the highly in vitro amyloidogenic TTR triple substitution mutant Gly53Ser/Glu54Asp/Leu55Ser. The structure of the protein was previously solved in the group (97), but reproducing the crystals proved impossible, thus preventing a more thorough characterization of the crystallized protein species. While not a direct aim of my thesis, finding a way to reproduce this batch has been a long term ambition during my studies.
RESULTS AND DISCUSSION

3. BINDING MODE DIFFERENCES FOR T₃ AND T₄ (PAPER I)

OBSERVATIONS FROM HUMAN AND PISCINE TTR (PAPER I AND UNPUBLISHED DATA)

Transthyretin has been identified in more than 20 vertebrate organisms (56). When this project was initiated, the evolutionary most distantly related transthyretin gene was that from the teleost fish *Sparus aurata* (sea bream), having 47% sequence identity with the human gene. A recent study identified TTR in *Petromyzon marinus* and *Lampetra appendix*, two species of lamprey, having a sequence identity of 36-47%. The lampreys are even more evolutionary distant from humans, belonging to the superfamily Euteleostei (57).

3.1. THE STRUCTURE OF SEA BREAM TTR

We solved the structure of sea bream TTR in its apo form at 1.75 Å resolution and in complexes with T₃ and T₄ at 1.9 Å resolution. These structures show that TTR from fish is very similar to TTR from human, rat and chicken. Superimposition of the main chain atoms of the ordered regions, comprising residues 12-98 and 104-123, yields a root mean square deviation of 0.71 Å. The main structural differences are located in the D-strand region; fish TTR lacks a proper D-strand and instead forms an extended loop that is stabilised through hydrogen bonding with water molecules not present in the human TTR structures. This is interesting, since the D-strand region has been suggested to be a hot-spot region for destabilising mutations (111), and structural alterations in this region was reported for the highly aggregation prone mutants Leu55Pro (112) and Gly53Ser/Glu54Asp/Leu55Ser mutants (97). However, no studies have yet been performed to assess the stability of the fish TTR.
Several X-ray crystallographic structures of TTR in complex with T4 have been solved for both human and rat wild type TTR (pdb code: 2ROX, (3); pdb code: 1ICT, (113); pdb code: 1IE4, (114)), all showing similar modes of binding. A comparison of the human complex structures with the sea bream TTR-T4 structure showed no major inter-species binding mode differences. A small shift of the ligand towards the hormone channel opening was however observed.

In the sea bream TTR-T3 complex structure a novel binding mode for thyroid hormones was revealed. As shown in figure 5b, this mode coordinates the outer ring iodine of T3, I3', to a position occupied by water in the apo protein, and the O4' atom of the hydroxyl group of T4. Such coordination is sterically impossible for T4, since the second outer ring iodine, I5', would clash with the exposed side chains of the hormone binding channel residues. A small fraction of the T3 also binds as T4, with the outer ring iodine coordinated in HBP2.

**Figure 5.** Omit difference maps of the hormone binding sites in fish TTR contoured at 5$. a) shows the binding mode of T4, while b) shows the binding modes of T3.
3.3. **T₃ BINDING IN HUMAN TTR**

For unknown reasons we were previously unable to crystallize the human TTR-T₃ complex. It was suggested that the recombinant expression system employed in the lab could be an explanation – the proteolytic processing in *E. coli* responsible for removing the starting methionine of expressed protein is not consistent and may result in a fraction of the monomers having an additional methionine at the N-terminus, a region known to be important for hormone binding (60). In obtaining the human TTR-T₃ crystals, we used protein that was expressed and purified to ensure homogenous N-termini. The complex crystallized isomorphously to apo TTR with a dimer in the asymmetric unit (see table 4), enabling the use of difference Fourier methods to solve the structure. As expected, significant ligand disorder was inferred through the asymmetric binding modes of T₃ along the 2-fold axis of the hormone binding channels, and strong signals in the electron density maps were observed only for the iodines. In omit Fₒ-Fₑ maps, density for conserved waters were visible from approx. 7σ, and for the iodines from approx. 13σ. Since the maps for the alanyl group and the ring structures of T₃ were very weak, the ligand was placed according to the location of the iodines. Figure 6 shows the location of T₃ in a superposition of the human and fish TTR molecules.

![Figure 6](image)

**Figure 6.** Omit Fₒ-Fₑ maps of the hormone binding sites of human TTR contoured at 6σ. The binding modes of T₃ in human TTR (blue) are similar to that of T₃ in fish TTR (yellow). a) Binding site 1 exhibits dual binding modes. One is similar to T₄ binding while the other is unique for T₃, as observed in fish TTR. b) T₃ in binding site 2 exhibits only the T₄ binding mode.
3.4. DIFFERENCES IN THYROID HORMONE PREFERENCE

In the fish TTR structure we observed an approximately 1 Å narrowing of the inner region of the hormone binding channels and a widening of the outer parts of the channel by 1-1.5 Å, compared to the human structure. A similar structural change is present in chicken TTR, which also preferentially bind T₃, suggesting that the shape of the channel is important for the hormone binding preference. These structural data correlate well with observations of the hormone binding differences between species, where the specificity for T₄ binding was suggested to be determined by the residues N-terminal of residue 10 (T₄ binding), and the T₃ binding by the channel core region (34,60,115). However, the substitutions responsible for the altered shape of the channel remain to be determined.

Table 4. Data collection and refinement statistics of the wtTTR-T₃ co-crystal structure. Values in parentheses are for the highest resolution shell

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<td>Unit cell parameters (Å, °) A, B, C</td>
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Refinement

| No. of reflections in working set   | 28701 (2042) |
| No. of reflections in test set      | 1530 (100)   |
| Residues included in model          | A10-A124, B10-B125, |
| No. of protein atoms refined        | 2153 |
| No. of solvent atoms                | 1868 |
| R-factor (%)                        | 19.1 (23.3) |
| Rfree (%)                           | 22.0 (25.4) |
| Average B factor (Å)                | 16.7 |
| R. m. s. d. bond lengths (Å)        | 0.020 |
| R. m. s. d. bond angles (°)         | 1.787 |
| Ramachandan plot analysis           | Most favoured regions (%) 91.0 |
| Additionally allowed regions (%)     | 9.0 |
4. AGGREGATION OF ATTR Y114C (PAPER II)

A severe TTR amyloidosis is caused by the Tyr114Cys substitution. It has been described in Japanese (116,117), and Dutch patients (118) and is primarily associated with CNS symptoms, mainly as a TTR associated form of cerebral amyloid angiopathy (CAA). The common clinical features of CAA are amyloid deposition in the cerebral blood vessels, cerebral haemorrhage, and rapidly progressive dementia. Patients may also suffer from vitreous opacities and cardiac disease.

4.1. TWO CYSTEINES PER MONOMER PROMOTE DISULFIDE MEDIATED AGGREGATION

Through the mutation, a second cysteine residue is gained in each TTR monomer. As cysteine is hydrophobic and a poor hydrogen bond donor compared to the substituted tyrosine, the mutation is likely to infer a destabilisation of the region. Also, Cys114 is solvent exposed and readily forms disulfide bonds to thiol containing molecules, possibly destabilising the protein even further (119). In contrast to the native TTR monomers, where other chemical bonding is necessary for aggregate formation, the dual cysteines of ATTR Tyr114Cys allow disulfide linked intermolecular networks and aggregates to form. Such aggregation has been suggested to be the reason for the mixed morphology of ex vivo amyloid from these patients; appearing as classical amyloid fibrils as well as amorphous aggregates. The occurrence of non-amyloid TTR deposits is not unique to Tyr114Cys patients, but such deposits are usually not congophilic (120), while the amorphous ATTR Tyr114Cys deposits are (119). Since Congo red has specificity for ordered beta-pleated sheets, the observed binding to the aggregates suggests an underlying order, perhaps as very short stretches of amyloid, that remain hidden within the aggregates (119).

4.1.1. CYS10 - A KEY RESIDUE IN TTR TETRAMER DESTABILISATION?

Chemical modifications of cysteine in TTR are very common in vivo and occur in healthy individuals as well as in TTR amyloidosis patients. The common modifications found in ex vivo TTR are cysteinylation, oxidation (Cys-SO$_{3}^-$), sulfonation (Cys-S-SO$_{4}^-$), glutathionylation, cysteinylglycylatation, and homocysteinylatation. Studies investigating the thermodynamic stability of conjugated tetramers suggest that sulfonation has a thermodynamically stabilising effect on the tetramers, while the binding of sulfhydryls decreases this stability (121-125). The underlying mechanisms for these observations remain to be found, but are likely to be related to the altered hydrophobic and hydrogen bonding patterns inferred by the modifications.
An elegant example of the in vivo destabilising effect of adduct conjugations was provided by studies on the TTR Val30Met variant, in vitro and in a rat model system. In vitro, there was no difference in the aggregation propensities between the Cys10 containing Val30Met protein and the Cys10Ser/Val30Met variant (126). In vivo, however, a significant amyloid deposition was observed in Val30Met rats but not in Cys10Ser/Val30Met rats (127). An in vitro follow-up study suggested that the discrepancy between the studies is most likely a consequence of post-translational conjugations of Cys10 in the rat. These protein modifications were shown to lower the tetramer dissociation energy to a level that enables amyloid formation during the short lifespan of a rat (128). Further stressing the importance of Cys10, amyloid in patients with the ATTR Val30Met variant has been reported to contain Cys10 disulfide linked protein (129).

4.1.2. TTR C10A/Y114C IS STRUCTURALLY UNCHANGED

Intrigued by the function of Cys10 in fibril formation of the Val30Met variant and the dual morphologies of the Cys114 ex vivo amyloid, we set out to investigate the role of Cys10 in the in vitro aggregation pathway of TTR Tyr114Cys. By substituting Cys10 to an alanine in the Cys10Ala/Tyr114Cys double mutant, thus eliminating the effect of Cys10 and any cooperative effects of the dual cysteines, we isolated the contribution of Cys114 to the amyloidogenic behaviour of the protein.

To ascertain that no unexpected structural changes were inferred by the Cys10Ala substitution, we solved the structure of the Cys10Ala/Tyr114Cys by X-ray crystallography to a resolution of 1.7 Å. Analysis of the structure did not reveal any major structural differences compared to the Tyr114Cys or the wild type protein. The reducing agent β-mercaptoethanol (BME), which was added to the crystallization solution, was found in the structure bound to Cys114. As observed in the Tyr114Cys structure, the binding inferred only minor conformational alterations compared to the wild type structure, mainly in the loop downstream of the α-helix in the same monomer.

4.2. DISULFIDE MEDIATED AGGREGATION OF TTR Y114C OCCURS SEQUENTIALLY

Based on the characteristics of the migration patterns of wild type TTR as well as the variants Cys10Ala/Tyr114Cys, Tyr114Cys and Val30Met by polyacrylamide gel electrophoretic (PAGE) methods, we suggest a sequential aggregation pathway of TTR Tyr114Cys. Early in the aggregation process, disulfide bonds between Cys114 residues are formed. As the aggregation process proceeds, possibly catalyzed by the destabilisation inferred through inter-Cys114 disulfide bonding, Cys10 becomes more exposed to the solvent and can form disulfides.
4.3. DISULFIDE LINKED DIMERS OF TTR C10A/Y114C CAN FORM FIBRILS IN VITRO

Through heating of the Cys10Ala/Tyr114Cys TTR, we produced an extensively disulfide linked protein that proved to be recognized by monoclonal antibodies specific for amyloidogenic TTR (described by Goldsteins et al. (130)). This protein readily formed protofibrils morphologically similar to those formed by the reduced ATTR Tyr114Cys variant (119) upon heating.

Based on these results, we suggest that fibril formation of ATTR Tyr114Cys is not dependent on Cys10, and also that fibrils may, but do not have to, consist of disulfide bonded protein. Dimers of TTR have previously been shown to form protofibrils in vitro (88,131), suggesting that native dimer interactions may be retained upon fibre formation. It should also be noted that our results do not rule out the possibility that inter-Cys114 disulfides may form between partially denatured tetramers as well as between dimeric and monomeric protein species. Several mechanisms by which fibril formation of the dimeric TTR Cys10Ala/Tyr114Cys as well as of monomeric and dimeric ATTR Tyr114Cys occurs are conceivable. Some of these are discussed below.

AGGREGATION OF DISULFIDE-BONDED PROTEIN

While sterically allowed, disulfide bonding between native-like TTR dimers is usually not observed in vitro or in vivo. This might reflect the low reactivity of the cysteines in the native dimers, but could also be a consequence of the thiol conjugation reactions occurring in plasma. The propensity for disulfide bond formation is increased in Cys114 compared to Cys10, and promotes formation of disulfide linked oligomers in ATTR Tyr114Cys. Whether these oligomers can actually form fibrils or if they are simply steps in the pathway of amorphous aggregation formation remain to be concluded, but, as we have shown in this study, they are clearly compatible with fibril formation.

Figure 7(i) shows a hypothetical fibril model of native-like dimers interconnected through Cys114-Cys114 disulfide bonds. A similar arrangement would be reasonable also for Cys10-Cys10 and Cys10-Cys114 linked dimers. Another possibility of accommodating disulfide linked protein in the fibrils is shown in Figure 7(ii). This is a more classical fibril formation model where the fibrils are stabilised by antiparallel β-strand interactions between the H- and A- or D-strands (Figure 7(ii)). Since the D-strand region has been shown to be very flexible and sometimes even non-existent in TTR structures (132), it may be argued that the β-strands A rather than the D-strands are of importance for fibril formation. To reflect this uncertainty, the D-strands potentially relevant for fibril formation were drawn as dashed lines.

Cys114-Cys114 linked monomers can only adopt a “wrong dimer” conformation, i.e., where the H-strands are positioned parallel with respect to each other. An “up-and-down” arrangement of these “wrong dimers” in a native antiparallel β-strand H
arrangement, as shown in Figure 7(iii), would appear to be compatible with fibre formation. This model for fibril formation is likely to be valid also for the Tyr114Cys and the Cys10Ala/Tyr114Cys variants assuming that Cys10-Cys114 interactions do not form to such an extent as to disrupt the fibril formation. These models are in agreement with a structural model suggested by Olofsson et al., where the major attractive forces within the fibrils are antiparallel β-strand interactions between the A-A’, B-B’ and F-F’ H-H’ strands (95). The model was based on the observation of the H/D exchange pattern of in vitro formed fibrils of TTR Tyr114Cys by NMR, and accommodates intermonomeric Cys114-Cys114 disulfides as well as Cys10-Cys10 disulfides provided a small shift of registry in the β-strands A, bringing these residues within bonding distance of each other.

Figure 7. Models for disulfide-bond mediated fibril formation of TTR Tyr114Cys. In (i), native-like (“true” dimers) aggregate through interdimeric Cys114 disulfide bonds while in (ii) through β-strand interactions in the edge-strand region. In model (iii), parallel H-H’-strand interactions (“wrong” dimers) form the fibrils, which are then further stabilized by intradimeric Cys114-Cys114 disulfides. β-Strand D is dashed to indicate that it might be absent in the
5. PROTEIN HEATING ENABLES PRODUCTION OF DIFFRACTION QUALITY CRYSTALS (PAPER III)

5.1. X-RAY CRYSTALLOGRAPHIC METHODS REQUIRE CRYSTALS

Employing the diffractive properties of a protein crystal to solve its three-dimensional structure is more and more becoming an automated process. The increasing availability and use of robotics in the crystallisation and data collection processes, and the continuing development of algorithms and expert systems for data processing and structure refinement has speeded up the structure solving process immensely. Combined with the contributions of the structural genomics initiatives in the fields of parallel cloning and expression screening, an impressive array of technological aids is available at the hands of the modern protein crystallographers. However, despite the technological achievements and clever tools, obtaining a structure by X-ray crystallographic methods is still a challenge. One of the major obstacles is to produce diffraction quality crystals.

The processes of nucleation and growth of protein crystals have been extensively studied and has been shown to depend on several parameters, the most important being the protein itself.

5.1.1. STRUCTURED, STABLE AND PURE

Ideal crystals are translational repeats of identical unit cells that grow through the formation of specific intermolecular interactions between the molecules – crystal contacts. To enable these interactions the protein has to be properly folded and homogeneous. Since a typical crystallization experiment lasts from days to weeks, the structure needs to have a high degree of structural integrity in solution and not degrade over time.

The chemical purity of the protein is important. There are several reasons for this; firstly, having a chemically defined and homogenous protein solution ensures that the crystals that are produced in a crystallization experiment consist of the protein target and not a contaminant. Secondly, if the proportion of contaminants is too high, it can disturb the formation of crystal contacts, thereby preventing crystal nucleation and growth. Thirdly, since protein crystals contain such a high degree of solvent, typically about 47% (133), contaminants easily become trapped in the crystal lattice, where they may disturb the order of the crystal lattice, thereby destroying the diffractive properties of the crystal or preventing further crystal growth.
5.1.2. HOMOGENOUS

As mentioned, the purity of the protein to be crystallized is important. However, the absence of foreign contaminating molecules alone does not suffice; the protein also needs to be homogenous.

Expression of different splice forms and post-translational modifications may yield a protein solution that is contaminated by itself. If such modifications affect the structural or biochemical properties of the protein, it may, despite being apparently pure, never crystallize. Yet another form of heterogeneity arises from the inherent flexibility of proteins, observed as different folding states, stabilities and surface properties. Through standard techniques such as affinity, ion-exchange and size exclusion chromatography isolating a protein from e.g. a crude *E. coli* lysate is fairly straightforward. Separating micro-heterogeneous forms of the same protein is however more problematic.

The TTR mutant TTR Gly53Ser/Glu54Asp/Leu55Ser was constructed, and proved to be highly prone to form fibrils *in vitro* (134). Despite a high degree of conformational heterogeneity and the presence of several aggregation forms in the solution, its structure was solved to 2.3 Å in year 2000 by Eneqvist et al. (97). A dramatic shift in the location of β-strand D was observed in the structure, and it was hypothesised that this rearrangement could be a general intermediate step in the amyloid forming process of amyloidogenic TTR mutants. Further studies of the crystallizable form of this protein were however hampered by our inability to reproduce the crystals, most likely due to the heterogeneity of the protein preparation.

To be able to further analyse the characteristics of the crystallizable species, we have evaluated different methods to overcome these problems.

5.2. RESULTS

Previously, the Gly53Ser/Glu54Asp/Leu55Ser mutant was expressed as inclusion bodies at 37°C. The inclusion bodies were then washed, solubilised in urea, and refolded prior to purification by ion exchange chromatography and size exclusion chromatography, eventually yielding a very aggregation prone protein (134). By lowering the induction temperature to 20°C and increasing the induction time to 15h, a soluble form of the protein was expressed. After optimising the chromatography protocols, we were able to isolate a relatively homogeneous and crystallizable form of the protein. However, probably due to incorporation of remaining contaminating heterogeneities, these crystals were very fragile, and did not diffract (figure 9a).

To further increase the homogeneity of the solution, we decided to try a modified version of the heat precipitation method suggested by Pusey et al. This technique exploits the difference in heat stability between properly folded and misfolded protein and has been used to improve the diffractive properties of crystals. Since native wild
type TTR has a unfolding temperature in the region of 100°C (22,68,80), we believed it to be a good candidate for heat-mediated precipitation of misfolded protein. The typical time-scale of the heating pulses described by Pusey and co-workers were in the ranges of seconds to minutes (135). In contrast, obtaining diffraction quality crystals of the Gly53Ser/Glu54Asp/Leu55Ser mutant required heat incubation for several days at 55°C. As shown in figure 8, the protein concentration decrease was initially quite rapid, but diminished over time and eventually reached a plateau after 48-72h. At this point, approximately 40% of the protein was precipitated.

The observed precipitation curve of the TTR Gly53Ser/Glu54Asp/Leu55Ser protein was notably different from that of the wild type protein, which did not precipitate to any significant extent during the experiment (data not shown), and suggests that the starting solution of the mutant protein was actually a mix of several folding intermediates with varying stabilities.

The resulting protein preparation was more homogenous, did not form the abundant aggregates reported previously, and readily formed well-diffracting crystals (see figure 9b). To verify that the protein was not adversely affected by the heating, the structure was solved, and was found to be highly similar to the previously published structure of this mutant. In contrast to the protocol developed by Pusey and co-workers, where short heat-pulses were employed as means of improving the quality of already diffracting crystals, we showed in this study that heating of a protein solution may actually be the difference between a successful structure determination project and failure.

![Figure 8. Protein concentration as a function of incubation time at 55°C.](image)
Figure 9. Crystals of TTR Ser53Gly/Asp54Glu/Ser55Leu formed by (a) non-heated protein, and (b) protein heated at 55°C for 48h.
CONCLUSIONS

In conclusion, the findings of this thesis provide the following:

A structural explanation for the difference in thyroid hormone preference between human and piscine transthyretin. Briefly, the thyroid hormone binding channel of piscine TTR has a wider entrance and a narrower cavity, resulting in a less efficient interaction with T₄, thereby increasing the relative affinity for T₃.

Evidence that the Cys10 residue of the disease related ATTR Tyr114Cys is not necessary for in vitro fibril formation, and that dimers formed through Cys114 disulfide bonds can form fibrils. An explanation of the manner in which the disease related ATTR Tyr114Cys undergoes disulfide mediated aggregation is also suggested.

A simple method for removing micro-heterogeneities from a protein solution, in which an increased solution temperature precipitates misfolded protein species out of solution.
Först vill jag uttrycka min stora tacksamhet till Liz för en utvecklande och stimulerande tid. Jag har verkligen uppskattat din entusiasm och ditt stöd och jag är glad för att jag fått möjligheten att jobba för dig.

Ett stort tack till alla er som har lärt mig (nästan) allt som är värt att kunna och lite till. Det mesta glöms ju bort under åren lopp, men ett par visdomar har faktiskt fastnat.


Anders Ö – Tack för dina synpunkter och förståeliga förklaringar om forskning, NMR, bakteriedodling och annat som gör livet komplicerat.

Carlos – Du är en inspirationsskälla och ett bra resesällskap – såväl till internationella konferenser som till centralhallen.

Christin – Nästan allt jag kan om praktisk proteinrening kan jag tack vare dig. Sedan har jag faktiskt rätt bra koll på dokumentation – också tack vare dig – men den kunskapen har jag inte tillämpat lika ofta…

Erik – Jag har smygläst din loggbok, länat dina bilder, din hiphop och en hel del av ditt tålamod. I gengäld har du fått stå ut med många år av norsk och finsk dödsmetall och eviga frågor om det mesta. Tack!

Jeanette – Tack vare dig har bioinformatiken blivit förståelig. Lycka till med allt!

Li-Xiao – Good luck with everything.

Malin – Tack för proteiner, pH-ställda buffertar, mass-körningar, och sena helg- och kvällsinducerings. Ge inte upp, en vacker dag kanske experimenten blir reproducerbara på första försöket. Ring mig då, så sätter vi alla rätt på introtävlingen…

Sheng-Hua – Thank you for sharing your time and knowledge with me.

Tobias – I value your expertise in the lab, and have enjoyed your company during data collections and conference trips.

Ulrika – Plötdigt händer det, min cyniska vän. Lycka till!

Uwe – Tack för givande diskussioner och datasamlingshjälp.
Ett stort tack till de nya och gamla gruppmedlemmarna Alex, Aaron, Andreas, Fredrik, Joel, Jonas, Lennart, Stefan, Talal, Teresa och Therese för en givande miljö och värdefulla diskussioner och synpunkter.

Karina – Jag lärde mig massor när vi delade kontor, tack för att du tog dig tid och för all hjälp under datasamlingarna.

Nina – Lycka till med metyleringarna och tandtrollsproteiner.

Maria – Du är en klippa! Tack för att du orkar klara allt från reseräkningar (som jag borde kunna fylla i själv) via konstiga beställningar till fantastisk älgfärs.

Anna-Mia – Tack för alla råd och tips om avhandlingsskrivande.


Magda-Lena – Du har oanade resurser av tålamod - att klara av att dela kontor med mig och min musiksmak i flera månader är imponerande.

Mina är på UCMP har varit intressanta och lärorika. Det har varit kul att jobba tillsammans med er alla och jag har lärt mig massor om sånt jag inte trodde fanns.

David, Niklas och Ali – Tack för alla råd och tips om antikroppssrening

I also want to thank Prof. Erik Lundgren and his labcrew, especially Gosia, Johan and Monika, for valuable discussions, help, advice and assistance with protein purification, antibody production and other things.

Mirjam & Magnus – när man börjar ha känt varandra mer än halva livet borde väl det mesta redan vara sagt, men jag som ibland är lite faordig har nog en del kvar… Tack för allt som varit – jag ser fram mot det som kommer!

Lunchdaternas Mats, Magnus och Erik – det har blivit lite g 1 e s a r e mellan luncherna det senaste året, men inte mindre trevligt.

Ett stort tack till Mamma & Pappa, David & Jenny, Anna & Jonas och de blivande (?) hockeyproffsen Ebba och Viggo för att ni uppmuntrar och tror på mig - trots att jag har varit rätt dålig på att förklara vad jag egentligen gör och har gjort.

Linda, Anny och Arvid

Tack för att ni finns

Jag älskar er

!!!
Ärftlig transthyretinamyloidos är en ovanlig och i allvarliga fall dödlig proteininlagringssjukdom som orsakas av mutationer i genen för transthyretin. Den kliniska bilden är huvudsakligen beroende av den bakomliggande genförändringen samt amyloidlokalisationen och -depositionshastigheten och omfattar vanligen neuropatier och myopatier av varierande grad. Det slutgiltiga målet med forskningsfältet som presenteras i denna avhandling är att förhindra eller bota transthyretinamyloidos. En förutsättning för att lyckas med detta ambitiösa mål är en ingående förståelse för proteinets grundläggande egenskaper, såväl i normalfallet som i de patologiska processerna, bland annat genom jämförande studier av humant och icke-humant transthyretin (TTR).

Den tredimensionella röntgenkristallografiska strukturen av TTR från fisken guldsparid (*Sparus aurata*) bestämdes till en upplösning på 1,75Å och befanns vara strukturellt snarlik humant TTR. Signifikanta skillnader observerades emellertid i och kring β-sträng D, en region som tros dissociera från huvudstrukturen innan själva bildningen av amyloid. Enligt denna hypotes leder D-strängsdissociationen till exponering av β-strängarna A och B, vilka därmed kan delta i de reaktioner som bildar amyloid. Under evolutionen har bindningspreferensererna för thyroideahormonerna T₃ (3,5,3’-trijod-L-thyronin) och T₄ (3,5,3',5'-tetrajod-L-thyronin) hos TTR ändrats. Humant TTR har högre affinitet för T₄ än för T₃, medan det motsatta förhållandet gäller för lägre vertebrater, t ex fisk, där T₃ är den huvudsakliga liganden. Strukturerna bestämdes för guldsparid i komplex med T₄ och med T₃ till 1,9 Å upplösning, samt för humant TTR i komplex med T₃ till 1,7 Å upplösning. Jämförande analyser visade på signifikanta skillnader i thyroideahormonbindningskanalen, vilken var vidare och grundare i fisk än i människa. Dessa strukturella skillnader kan delvis förklara olikheterna i hormonbindning mellan högre och lägre vertebrater.


Vi har också modifierat och anpassat en metod för uppvärmning av proteiner för att möjliggöra kristallisation i ett synergen problematiskt proteinkristallisations-experiment. Genom uppvärmning av proteinlösningen lyckades vi separera olika former av mikroheterogeniteter från det rättveckade proteinet, som sedan bildade kristaller av god röntgendiffraktiv kvalitet.
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