Transthyretin from a structural perspective

av

Andreas Hörnberg

Akademisk avhandling

som med vederbörligt tillstånd av rektorsämbetet vid Umeå Universitet för avläggande av filosofie doktorsexamen framläggs till offentligt förvar i Major Groove, Institutionen för Molekylärbiologi, byggnad 6L, fredagen den 27 februari, kl 10.00.

Avhandlingen kommer att förvaras på engelska.

Fakultetsopponent: Prof. Hugo L. Monaco, Department of Science and Technology, University of Verona, Italy.
Title
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Abstract
Conformational changes in human proteins can induce several types of diseases. The nature of the conformational changes is largely unknown, but some lead to amyloid fibril formation. Amyloid fibrils accumulate in the extra-cellular space of tissues resulting in disruption of organ function. Transthyretin (TTR) is a plasma protein involved in three amyloid diseases, familial amyloidotic polyneuropathy, familial amyloidotic cardiomyopathy, and senile systemic amyloidosis. The latter disease involves conformational changes in the wild-type structure of the protein, whereas the others are caused by a gene mutation.

Our goal is to increase the knowledge of why and how some proteins aggregate into amyloid fibrils by solving and analyzing structures of different TTR variants of which some can form amyloid fibrils, whereas others cannot. The crystal structures of wild-type TTR and many of its disease-causing mutants have previously been determined, and observed structural discrepancies between mutant and wild type were claimed to be of importance for amyloid formation. We performed a comparative analysis of all, at that point, known structures of TTR. As a reference for our study, we determined a 1.5 Å resolution structure of human wild-type TTR. We found that the previously reported structural differences between wild-type and mutant TTR were insignificant and did not provide clues to the mechanism for amyloid formation.

We showed the double mutant TTR-Ala108Tyr/Leu110Glu to be less amyloidogenic than wild-type transthyretin. Since the structure of few non-amyloidogenic mutants are known, we solved its structure in two space groups, C2 and P2₁2₁2, where the latter was consistent with most of the structures of transthyretin. Only the highly amyloidogenic mutant ATTR-Leu55Pro has previously been solved in C2. The packing of molecules in our C2 crystal was close-to-identical to the ATTR-Leu55Pro crystal structure, ruling out the described ATTR-Leu55Pro packing interactions as significant for amyloidosis. The C2 structure displayed a large shift in residues Leu55-Leu58, a structural change previously found only in amyloidogenic TTR variants. Combined with previous data, this suggests that transthyretin in solution contains a mixture of molecules with different conformations. This metastability of transthyretin provides insight to why some proteins aggregate into amyloid fibrils.

The natural ligand thyroxine has been shown to stabilize TTR. Small molecules, based on thyroxine, with the potential to serve as inhibitors for amyloid fibril formation are under development. Iodine is a component of thyroxine and we found that TTR also bound free iodide ions. Taking advantage of the anomalous scattering of iodide, we solved the iodide-bound TTR structure using the single-wavelength anomalous dispersion method. In addition, we determined the TTR-chloride structure. Both chloride and iodide stabilized transthyretin where iodide stabilized better. From the thyroxine-TTR structure, three halogen-binding pockets have been identified in each TTR monomer. We found three bound iodides per TTR monomer, two of which were in the thyroxine-binding channel. This indicates that only two of the three halogen-binding pockets in the thyroid-hormone binding channel are optimal for halogen binding. Our results might be useful for the continuing design of small molecule ligands, which in the end can lead to inhibitors for amyloid diseases.

Keywords: transthyretin, X-ray crystallography, protein structure, amyloidosis, structural comparison, anomalous diffraction.
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Table of contents

Abstract..................................................................................................................... 6
Publications................................................................................................................ 7
Abbreviations............................................................................................................. 8
Introduction............................................................................................................... 9
Amyloidosis............................................................................................................. 9
  Historical perspective.......................................................................................... 9
  Medical disorders caused by amyloidosis......................................................... 9
  Similar fibril structure from proteins with different native fold....................... 11
  Conformational changes mediate amyloid formation......................................... 12
  Is amyloid responsible for disease?.................................................................... 12
Transthyretin......................................................................................................... 13
  TTR is a transport protein ............................................................................... 13
  The X-ray structure of transthyretin and its complexes with natural ligands. 15
  TTR-associated amyloidosis ........................................................................... 17
  Current models for TTR amyloid formation .................................................... 19
  Stabilization of the tetramer inhibit amyloid formation.................................. 19
  Small molecule inhibitors................................................................................. 21
Aim ......................................................................................................................... 22
Results and discussion ............................................................................................ 23
  Structural study of transthyretin structures (Paper I)......................................... 23
    Do structures of transthyretin determined at low pH represent the structure at
    physiological pH?........................................................................................... 24
    No structural evidence for TTR amyloidogenesis from the 23 analyzed
    structures ........................................................................................................ 24
    Recently published structures......................................................................... 26
  Flexibility of β-strand D (Paper II)................................................................ 28
    TTR-A108Y/L110E is more stabile and less amyloidogenic than wild type. 28
    Two structures of TTR-A108Y/L110E .......................................................... 29
    Metastability of β-strand D ........................................................................... 29
    Packing interactions of TTR-A108Y/L110E in space group C2..................... 31
  The effect of iodide on stability and structure of transthyretin (Paper III).... 31
    Iodide stabilizes the native fold of transthyretin ........................................... 32
    The structure of iodide-bound TTR was solved by the SAD method........... 32
    The structure of iodide-TTR and chloride-TTR and positioning of the ions . 32
Conclusions............................................................................................................. 35
Acknowledgement .................................................................................................. 36
References............................................................................................................... 38
Abstract

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Publications

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


Structure coordinates from this thesis can be found at the RCSB protein data bank, accession code 1F41.
**Abbreviations**

Aβ  Alzheimer’s beta peptide
ATTR-AXXB  Amyloidogenic variant of transthyretin, mutated from amino acid A to B in position XX (e.g. Val30Met where valine is replaced by methionine in position 30).
B-factor  Temperature factor, a measure of individual atoms mobility.
BME  β-mercaptoethanol
CNS  Central nervous system
CSF  Cerebrospinal fluid
DIT  Diiodotyrosine
FAC  Familial amyloidotic cardiomyopathy
FAP  Familial amyloidotic polyneuropathy
GAGs  Glycosaminoglycans
MIT  Moniodotyrosine
MME  Monomethyl ether
PEG  Polyethylene glycol
RBP  Retinol-binding protein
R.m.s.  Root mean square
SAD  Single-wavelength anomalous dispersion
SSA  Senile systemic amyloidosis
T₃  3,5,3'-triiodo-L-thyronine
T₄  3,5,3',5'-tetraiodo-L-thyronine, thyroxine
Tg  Thyroglobulin
TTR  Transthyretin
TTR-A108Y/L110E_p21212  TTR-A108Y/L110E structure of transthyretin solved in space group P21212
TTR-A108Y/L110E_c2  TTR-A108Y/L110E structure of transthyretin solved in space group C2
Introduction

Amyloidosis

Amyloidosis is a group of diseases in which normally soluble proteins undergo a conformational change and aggregate into amyloid fibrils. Accumulation of these amyloid fibrils in the extra-cellular space of tissues leads to tissue damage and ultimately to disruption of organ function (for reviews on amyloidosis see Tan and Pepys, 1994; Rochet and Lansbury, 2000; Adams, 2001; Pepys, 2001).

Historical perspective

The term amyloid was first used in 1854 by the German physician Rudolph Virchow when he observed an anomalous tissue, which stained blue upon addition of iodide (Virchow et al, 1854). The iodide-staining method was mainly used to stain starch and cellulose, he thereby concluded that the deposits contained carbohydrates and named the tissue “corpora amylacea” from the Greek and Latin words for cellulose amylym and amylon, respectively. Five years later, it was discovered by Friedreich and Kekule that, due to their high nitrogen content, the amyloid deposits mainly contained protein rather than cellulose (reviewed by Sipe and Cohen, 2000). In the late 1920’s, a green birefringence was observed when the amyloid was stained with the dye Congo red using a polarized light microscope (Divry and Florkin, 1927) and Congo red is still used today as a preliminary diagnostic assay for detecting amyloid fibrils. At this time, however, no clues of the underlying structure of the deposits were known. Amyloid from a patient with amyloidosis in spleen, liver and kidney was later shown to have fibrillar structure by electron microscopy (Cohen and Calkins, 1959). A few years later Pras and co-workers developed a method for purification of fibrils from tissues based on their high solubility in pure water (Pras et al., 1968). By analyzing these fibrils, it was concluded that each clinical syndrome was derived from a specific protein. One year later, another important historical finding was made with X-ray diffraction. Diffraction of isolated amyloid fibrils showed that all fibrils examined had an ordered so-called cross-beta structure (Bonar et al., 1969), in which the polypeptide backbone takes the form of β-strands organized predominantly perpendicular to the fibril axis.

Medical disorders caused by amyloidosis

Amyloid diseases belong to a broader defined group called protein-aggregation diseases. This group includes disorders characterized by intracellular amyloid-like aggregates, with Huntington’s and Parkinson’s diseases and familial amyotrophic
lateral sclerosis being the most well known (Johnson, 2000). These non-amyloid disorders will not be discussed further in this thesis.

Table 1 Amyloid proteins associated with human diseases

<table>
<thead>
<tr>
<th>Protein</th>
<th>Clinical syndrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Amyloid A</td>
<td>Secondary systemic amyloidosis</td>
</tr>
<tr>
<td>Amyloid-β Precursor Protein</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ABriPP</td>
<td>Familial British dementia</td>
</tr>
<tr>
<td>Cystatin C</td>
<td>Hereditary cerebral hemorrhage with amyloidosis, Icelandic type</td>
</tr>
<tr>
<td>ADan</td>
<td>Familial Danish dementia</td>
</tr>
<tr>
<td>α-fibrinogen</td>
<td>Hereditary renal amyloidiosis</td>
</tr>
<tr>
<td>Immunoglobulin heavy chain</td>
<td>Primary myeloma-associated amyloidosis</td>
</tr>
<tr>
<td>Insulin</td>
<td>Injection localized amyloidosis</td>
</tr>
<tr>
<td>Islet amyloid polypeptide (amylin)</td>
<td>Type II diabetes mellitus</td>
</tr>
<tr>
<td>Immunoglobulin light chain (κ and λ)</td>
<td>Primary myeloma-associated amyloidosis</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>Hereditary systemic amyloidosis</td>
</tr>
<tr>
<td>Lactadherin (medin)</td>
<td>Aortic medial amyloid</td>
</tr>
<tr>
<td>Atrial natriuretic factor</td>
<td>Cardiac atria</td>
</tr>
<tr>
<td>Apolipoprotein A-I</td>
<td>Familial amyloidotic polyneuropathy-like disease</td>
</tr>
<tr>
<td>Apolipoprotein A-II</td>
<td>Hereditary renal amyloidiosis</td>
</tr>
<tr>
<td>Apolipoprotein A-IV</td>
<td>Senile systemic amyloidiosis (co-deposition with transthyretin)</td>
</tr>
<tr>
<td>Prion protein</td>
<td>Creutzfeldt-Jakob disease</td>
</tr>
<tr>
<td>Transthyretin</td>
<td>Familial amyloidotic polyneuropathy</td>
</tr>
<tr>
<td>β2-microglobulin</td>
<td>Familial amyloidotic cardiomyopathy</td>
</tr>
<tr>
<td>Procalcitonin</td>
<td>Senile systemic amyloidiosis</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>Dialysis related amyloidiosis</td>
</tr>
<tr>
<td>Keratin</td>
<td>Medullary carcinoma of the thyroid</td>
</tr>
<tr>
<td>Prolactin</td>
<td>Familial subepithelial corneal amyloidosis</td>
</tr>
<tr>
<td>Kerato-epithelin</td>
<td>Primary localized cutaneous amyloidosis</td>
</tr>
<tr>
<td></td>
<td>Prolactinoma with spherical amyloid deposition</td>
</tr>
<tr>
<td></td>
<td>Lattice corneal dystrophy</td>
</tr>
</tbody>
</table>

Amyloid is defined as extra-cellular protein deposits with fibrillar structure that have the ability to bind the dye Congo red. Currently there are more than 20 different proteins known to precipitate as amyloid, resulting in diseases of which
the most well known are Alzheimer’s disease and Creutzfeldt-Jakob disease caused by the Alzheimer’s beta peptide (Aβ) and the prion protein, respectively (see a recent review by Ghiso and Frangione, 2002). The amyloid proteins and their clinical syndromes are listed in Table 1. New proteins are identified every year of which the most recent is lactoferrin (Ando et al., 2002). In 1997, only 17 human proteins were known aggregate into amyloid fibrils and today there are 26.

**Similar fibril structure from proteins with different native fold**

As previously mentioned, all amyloid fibrils display a green birefringence in polarized light when stained with Congo red (Puchtler et al., 1962). In addition, they display a characteristic spectral red shift in fluorescence when interacting with thioflavin T (LeVine, 1993). Other characteristics for amyloid fibrils are their similar physical appearance in electron microscopy and atomic force microscopy (AFM). They appear as straight, rigid and unbranched fibrils about 70 to 140 Å in diameter and with a length of 1,200-16,000 Å (Cohen and Calkins, 1959; Cohen and Shirahama, 1973; Serpell et al., 2000). Electron microscopy on *ex vivo* fibrils has also shown that amyloid is built up from a defined number (4-6) of protofilaments, depending on the protein, to give rise to the mature fibril (Serpell et al., 2000).

Amyloid fibrils have been studied with synchrotron X-ray fiber diffraction and the core structure of amyloid fibrils has been suggested to be similar for a wide range of disease-causing protein aggregates with different native structure, e.g. insulin, Aβ, prion protein, amyloid A, immunoglobulin light chain, transthyretin, apolipoprotein A1, lysozyme and islet amyloid polypeptide (Burke and Rougvie, 1972; Kirschner et al., 1986; Nguyen et al., 1995; Sunde et al., 1997). X-ray diffraction of the amyloid fibers gives a pattern with a meridional reflection at 4.7-4.8 Å and an equatorial reflection at around 10 Å. The reflections in the diffraction pattern arise from a regularly ordered repeat within the fibril, where 4.7-4.8 Å is the approximate distance between two β-strands and the reflection at around 10 Å indicates the distance between two β-sheets, with the distance depending on the composition of the side-chains (Fig. 1, Kirschner et al., 1986; Nguyen et al., 1995; Sunde et al., 1997). This pattern is interpreted as a cross-β structure in which most of the polypeptide chain of the proteins is organized in β-strands, arranged perpendicular to the axis of the fibril (Pauling and Corey, 1951; Bonar et al., 1969).

The protein serum amyloid P component (SAP; Skinner et al., 1974) is universally present in all amyloid deposits (as reviewed by Pepys et al., 1997). In the presence of calcium, SAP is remarkably resistant to proteolytic degradation and it has been shown in *vitro* that SAP can prevent proteolysis of fibrils from Alzheimer’s disease, systemic amyloid A amyloidosis and systemic monoclonal light chain amyloidosis (Tennent et al., 1995). It is therefore likely that SAP has
the same function on all amyloid fibrils in vivo. As SAP was shown not to function as an enzyme inhibitor and was only protective when bound to the fibrils, the protection is probably mediated by coating the fibrils by SAP molecules (Tennent et al., 1995). Other components of less known function but connected to all or almost all fibrils are apolipoprotein-E (Namba et al., 1991; Wisniewski and Frangione, 1992; Gallo et al., 1994) and sulfated glycosaminoglycans (GAGs), specifically heparan sulfate, dermatan sulfate and chondroitin sulfate (Magnus et al., 1989; Nelson et al., 1991; Kisilevsky, 2000).

![Figure 1. Proposed arrangement of the β-strands and sheets in the fibril as evaluated by X-ray fibril diffraction. The distance between two β-strands is ~4.8 Å and the distance between two independent sheets is approximately 10 Å.](image)

**Conformational changes mediate amyloid formation**

It is generally accepted that a conformational change or protein cleavage is necessary for fibril formation to occur (Kelly, 1997; Kelly, 1998). To date, the conformational change needed is not known for any amyloidogenic protein. Since all amyloid fibrils have similar structural features, there could be a similar mechanism of fibril formation. The effort to understand the mechanism for fibrillogenesis has increased in recent years, with the aim of developing small molecule inhibitors needed for medical cures (Peterson et al., 1998; Hammarström et al., 2003b).

**Is amyloid responsible for disease?**

It has been shown, at least for transthyretin and Alzheimer’s β-peptide, that the amyloid fibrils themselves are not toxic to the cells. Rather, toxicity is developed from small soluble oligomeric aggregates (Lambert et al., 1998; Sousa et al., 2001; Andersson et al., 2002), which have been shown to be toxic to cells in a free-radical dependent manner that can be inhibited by anti-oxidants (Behl et al., 1994; Andersson et al., 2002). Amyloid fibrils have been proposed to have a protection
mechanism by storing the misfolded proteins in non-toxic fibrils and thereby removing toxic protofilaments from circulation (Andersson et al., 2002).

**Transthyretin**

Transthyretin (TTR) is one of the 26 proteins known to cause amyloid disease. The transthyretin gene has been found on the long arm (q) of chromosome 18 in the region 18q11.2-q12.1 (Sparkes et al., 1987) and consists of four exons and three introns spanning about 7 kbp (Tsuzuki et al., 1985). The homotetrameric protein (molecular weight of about 54 kDa in solution (Branch et al., 1971)), with 127 amino acids per monomer, is mainly produced in the liver but also in the choroid plexus of the brain and in the eye (Felding and Fex, 1982; Herbert et al., 1986; Mita et al., 1986; Inada, 1988).

Normal plasma concentration of transthyretin in adults is around 20 mg/100ml serum (Stabilini et al., 1968). A depression of this level is significant when the liver is participating in the acute phase response to injury or during conditions of malnutrition or chronic inflammation (Dickson et al 1982).

**TTR is a transport protein**

Originally, transthyretin was called prealbumin because it ran ahead of albumin on a serum protein gel electrophoresis (this is true for the human protein, but not for the bovine protein). Since the protein has been shown to transport both thyroid hormones (3,5,3',5'-tetraiodothyronine (thyroxine, also called T4, Fig. 2A) and 3,5,3'-triiodothyronine (T3, Fig. 2B, Ingbar, 1958)) and retinol in complex with retinol-binding protein (RBP, Kanai et al., 1968) the name was changed to transthyretin after a compromise by the early TTR researchers, Drs J. Robbins, D. Goodman, T. Peters and H.G. Schwick (Nomenclature Committee of IUB, 1981).

![Figure 2. Structures of A) 3,5,3',5'-tetraiodothyronine (thyroxine, T4) and B) 3,5,3'-triiodothyronine (T3).](image)

The retinol-binding protein was shown to have no effect on the affinity and binding capacity for thyroxine (Raz and Goodman, 1969). Knockout of the mouse
transthyretin gene did not result in any abnormality in fetal development or life span of animals (Episkopou et al., 1993). The homozygous animals had no detectable plasma levels of vitamin A, but did not show any sign of vitamin-A deficiency. Total T4 levels in plasma were also reduced in the knockout mice, without any morphological difference in the thyroid (Episkopou et al., 1993).

**Thyroid hormone transport**

The main secreted hormone of the thyroid gland is T4. Deiodination of the outer ring result in the T3 hormone, which has a much higher affinity for the thyroid hormone receptors (Darras et al 1998). TTR together with two other proteins, albumin and thyroxine-binding globulin, are responsible for transport of T4 with the latter being the major serum transporter (75% of all T4 molecules). Transthyretin is, however, the major transport protein in the central nervous system (CNS), where most of the protein-bound T4 is bound to TTR (Hagen and Elliot, 1973). Each transthyretin tetramer has two T4-binding sites, but due to negative cooperativity only one of them is occupied at physiological conditions (Ferguson et al, 1975). It has, however, been shown in vitro that with an excess of thyroxine (4:1, T4: TTR), TTR with two bound thyroxine molecules was the major complex and with an increased excess (5:1) only the fully saturated complex was observed (McCammon et al., 2002).

Some of the target genes for T3 are growth hormone, osteocalcin, malic enzyme, TSH and the cerebellum genes; calbindin, IP3 receptor and myelin basic protein (As reviewed by Brent, 2000). This gene regulation of T3 leads to involvement in normal growth and development of the central nervous system, small intestine and bone (Brent, 2000).

**Iodide a necessary precursor for thyroxine anabolism**

The precursor molecules of thyroxine are circulating iodide and tyrosyl residues in the thyroglobulin molecule. Iodide is transported into the thyroid gland by the sodium-iodide symporter (NIS, for a review see De la Vieja et al., 2000), where it is oxidized and attached to tyrosyl residues in thyroglobulin (Tg) to make mainly monoiodotyrosine (MIT) and diiodotyrosine (DIT, Ogawara et al, 1972). Within the Tg molecule, coupling of two DIT residues produces thyroxine in a process catalyzed by thyroid peroxidase (Lamas et al., 1972). T3 is similarly produced by coupling one MIT and one DIT molecule. The thyroglobulin molecules are subsequently degraded by the endopeptidases cathepsins B, L and D, and exopeptidases (Dunn et al., 1991a; Dunn et al., 1991b; Dunn et al., 1996). This ultimately results in synthesis of T4 and T3 which then are released from the cell. Through stimulation of transcription factors and increased activity of cathepsins B and L, the thyroid-stimulating hormone (TSH) is involved in most of the aspects of iodine metabolism in the thyroid organ including increased production of Tg,
thyroperoxidase, and NIS (see review by Dunn and Dunn, 2001). For a good review on disorders resulting from lack of iodide, see Delange (1994).

**Retinol-loaded RBP transport**

The retinol-binding protein is a 21 kDa (183 amino acids) molecule. The retinol molecule is bound to the highly hydrophobic core of RBP. RBP would be rapidly eliminated from plasma by glomerular filtration if it was not further bound to transthyretin (Kanai et al., 1968). The binding of TTR to RBP increases the affinity of RBP for retinol and shields retinol from the environment better than when retinol is bound to RBP alone (Goodman and Raz, 1972). Two naturally occurring mutations (TTR-Ile84Asn and TTR-Ile84Ser) prevent TTR-RBP complex formation and individuals carrying these mutations have substantially lowered RBP plasma concentrations (Waits et al., 1995). These mutants are also known to aggregate and form transthyretin-related amyloid (familial amyloidotic polynuropathy, Dwulet and Benson, 1986).

**The X-ray structure of transthyretin and its complexes with natural ligands**

The structure of transthyretin was first solved by Prof. Colin Blake in 1978 and many TTR structures of wild types, mutants and ligand complexes have since then been solved and their coordinates deposited in the Research collaboratory of structural bioinformatics protein data bank (RCSB, http://nist.rcsb.org/pdb, Blake et al., 1978; Ciszak et al., 1992; Wojtczak et al., 1992; Hamilton et al., 1993; Steinrauf et al., 1993; Wojtczak et al., 1993; Monaco et al., 1995; Zanotti et al., 1995; Damas et al., 1996; Wojtczak et al., 1996; Peterson et al., 1998; Schormann et al., 1998; Sebastiāo et al., 1998; Naylor and Newcomer, 1999; Klabunde et al., 2000; Eneqvist et al 2001, Wojtczak et al., 2001; Eneqvist et al., 2002; Paper I).

The TTR structure consists of eight beta strands and one short alpha helix. The strands form two sheets, comprising β-strands D-A-G-H and C-B-E-F, into a β-barrel structure. Dimerization occurs mainly between the main chain atoms of the two H-strands (residues Ser115-Thr123) resulting in an eight stranded β-sheet (Fig. 3A). The contact region between the dimers, which produces the functional tetramer, is small and consists of hydrophobic interactions between the AB loop and the GH loop. This produces a hydrophobic pocket between the two dimers that has been shown to be the binding site for thyroxine (Fig. 3B, Wojtczak et al 1996).

Each of the hormone-binding sites of the TTR tetramer contains three pairs of halogen-binding (HB) pockets (P1-P1*, P2-P2* and P3-P3*, where * indicates the symmetry related halogen-binding pocket (Wojtczak et al., 1996)). The outer HBP1 pocket is formed by the side chains of Met13, Lys15 and Thr106, while the middle pocket HBP2 is surrounded by side chains of Lys15, Leu17, Ala109 and Ala110 with carbonyl groups of Lys15, Ala108 and Ala109 forming a hydrophilic
surface. The innermost pocket (HBP3) is defined by the side chains of Ala108, Leu110, Ser117, and Thr119, with a hydrophilic surface constituting the main chain carbonyl oxygen and amino groups of Ala108, Ala109, Leu110, Thr118 and the side chains of Ser117 and Thr119. The space group for most of the solved transthyretin molecules has a two-fold crystallographic axis at the thyroxine-binding pocket. Thyroxine, however, is an asymmetric molecule (Fig. 2A) and therefore positioned in two orientations, which prevent precise description of its protein-binding interactions. In the complex between TTR and T4, the four iodines are positioned in the HBP1 (SA128 I5), HBP2 (SA128 I3'), HBP2* (SA128 I3) and HBP3 (SA128 I5') site (pdb code: 2ROX, Wojtczak et al., 1996).

![Figure 3](image_url)

Figure 3. The three-dimensional structure of transthyretin displayed as A) a dimer and B) a tetramer in complex with thyroxine and C) retinol-binding protein.
The structure of RBP is composed of a short α-helix and nine β-strands which build a β-barrel with six strands in each of the two orthogonal β-sheets. This arrangement is made possible by the fact that three strands (A, E and F) are shared between the two sheets (strands A-B-C-D-E-F and E-F-G-H-A-I, respectively (pdb code: 1RBP, Cowan et al., 1990)). The retinol is encapsulated in the binding cavity with the ring structure of the retinol innermost. This makes only the hydroxyl moiety of the retinol solvent accessible in this protein-ligand complex (Cowan et al., 1990). The structure of the retinol loaded TTR-RBP complex has been solved (Monaco et al., 1995; Naylor and Newcomer, 1999). Two molecules of RBP bind to the transthyretin tetramer (Fig. 3C). The main interaction area comprises hydrophobic residues from the α-helix (residues 80-86) of TTR to the loop between β-strands 5 and 6 (residues 63-67), and the loop region between β-strands 7 and 8 in the RBP molecule. In one of the two RBP molecules from the all human TTR-RBP complex (pdb code: 1qab), the carboxy-terminus is defined and interacts with TTR resulting in 40% increase of total buried surface area in the RBP-TTR interface. The significance of the C-terminal end for TTR binding is also illustrated by a truncated form of RBP found in urine which does not contain vitamin A and lacks the C-terminal lysine. This truncated vitamin A-free RBP molecule has no affinity for TTR (Rask et al., 1971).

**TTR-associated amyloidosis**

There are three known diseases caused by transthyretin; senile systemic amyloidosis (SSA), familial amyloidotic polyneuropathy (FAP) and familial amyloidotic cardiomyopathy (FAC, for reviews on TTR amyloidosis see Saraiva, 1995; Benson and Uemichi, 1996; Hamilton and Benson, 2001). There are today more than 80 single point mutations of TTR identified and most of them are known to cause disease (Connors et al., 2000; Eneqvist and Sauer-Eriksson, 2001). The mutations are distributed all over the transthyretin sequence and structure, but with a “hot spot” in the edge strand area (residues 45-58) defined by the C-strand, CD-loop, D-strand and in a part of the DE-loop which all contain amino acids more prone to aggregate into amyloid fibrils (Serpell et al., 1996; Eneqvist and Sauer-Eriksson, 2001). Interestingly, the wild-type protein is also known to aggregate into amyloid fibrils (Westermark et al., 1990).

There are also mutations that suppress amyloid formation but only if the mutations are situated on different alleles. For example, patients with Val30Met on one allele and Thr119Met on the other allele are much less affected by disease than patients with the Val30Met mutation alone (Coelho et al., 1993; Coelho et al., 1996; Longo Alves et al., 1997).
Senile systemic amyloidosis

Transthyretin is the main component of the disorder senile systemic amyloidosis (previously called senile cardiac amyloidosis), where the amyloid has been shown to be composed of the wild-type protein (Westermark et al., 1990). It is an abundant disease affecting ~25% of the population over 80 years of age (Pomerance, 1966; Cornwell et al., 1983). The amyloid fibrils are systemically located in many organs, but are mostly concentrated to the heart (Pitkänen et al., 1984). Usually the deposits are benign, but some individuals (mainly men) are affected with heavy deposits in the heart giving rise to congestive heart failure (Pomerance, 1966). Taking into account that wild-type transthyretin can, with time, form amyloid fibrils emphasize the need for additional mechanisms besides amino acid substitution that are important for fibrillogenesis.

Familial amyloidotic polyneuropathy

In 1952, the Portuguese Physician Corino Andrade observed several patients with what he described as “a peculiar form of peripheral neuropathy” (Andrade, 1952). This included degeneration of the myelin sheaths in peripheral nerves due to amyloid deposits, diminished volume of all muscles, sexual impotence and gastrointestinal troubles: diarrhea, colic and indigestion and sphincter disturbances. The patients also had less sensibility in the feet and legs, with their feet being insensitive to heat, cold and pain (Andrade, 1952). These are typical symptoms of the disease we now call FAP, which is characterized mainly by peripheral neuropathy, but also by vitreous deposits, gastrointestinal dysfunction, carpal tunnel syndrome (pain, numbness, tingling in the hands) and heart conduction disturbances (as reviewed by Benson and Uemichi, 1996). A quarter of a century after Andrade’s findings, it was shown that transthyretin was the main constituent of the fibrillar deposits (Costa et al., 1978). The first mutant associated with FAP, ATTR-Val30Met, which is also the most common mutation found in FAP patients living in the north of Sweden (Skellefteå, hence it is called ‘Skellefte-sjukan’ in Sweden) was reported in 1983 (Dwulet and Benson, 1983; Tawara et al., 1983). FAP is a hereditary disease found worldwide, but it is more prevalent in certain areas of Sweden, Portugal, Japan and Brazil (for reviews see Buxbaum and Tagoe, 2000; Suhr et al., 2003).

Even though the liver is not the site of amyloidosis it produces the transthyretin protein and when a liver producing the ATTR-Val30Met mutant is replaced with a liver producing wild-type protein, it leads to a complete disappearance of variant TTR in the blood stream (Holmgren et al., 1991). Currently, liver transplantation is the only existing treatment of FAP and all of the analyzed patients reported an arrest in the development of disease (Holmgren et al., 1993). In a long-term follow-up study it was shown that the age of the patient at the time for the liver transplant
and the nutritional status of the patient are two important factors for survival, with underfed patients having a low survival rate (Suhr et al., 2002).

**Familial amyloidotic cardiomyopathy**

Transthyretin amyloid deposits are not always localized to peripheral nerves, as in the case of FAP. Some mutations in the TTR gene cause heavy deposits of amyloid fibrils in the heart resulting in cardiac failure. The disease where TTR amyloid fibrils are concentrated to the heart is called (FAC). ATTR-Val122Ile, which represents the most common of all transthyretin variants, causes a disorder of the FAC type. It is estimated that around 3-4% of the African-American population is heterozygous for this variant (Jacobson et al., 1996; Afolabi et al., 2000). In *vitro* experiment shows that ATTR-Val122Ile destabilizes the transthyretin tetramer, resulting in a monomer, which self-assembles into amyloid fibrils at a higher rate than wild-type protein (Jiang et al., 2001a). This may explain why patients with ATTR-Val122Ile have an increased risk for cardiac amyloid depositions compared to people with wild-type TTR (higher penetrance of disease, Jacobson et al., 1997).

FAC mutants found in Scandinavia include ATTR-Leu111Met (Denmark) with an onset of disease at about 40 years of age with progression to death in 3 to 6 years, and ATTR-Ala45Ser (Sweden), which is characterized by a slowly progressing cardiomyopathy with a late onset (Frederiksen et al., 1962; Ranlov et al., 1992; Janunger et al., 2000).

**Current models for TTR amyloid formation**

There are many models for the amyloid formation of transthyretin, and the most widely accepted is one where the transthyretin tetramer is disrupted into monomers (McCutchan and Kelly, 1993; Lai et al., 1996; Kelly, 1997; Kelly, 1998; Nettleton et al., 1998; Quintas et al., 1999; Redondo et al., 2000; Jiang et al., 2001b; Quintas et al., 2001). These native monomers are believed to undergo a conformational change into an so called amyloidogenic monomer, which then form fibrils. There exist also other models in which the building block is a either dimer (Olofsson et al., 2001; Serag et al., 2001; Serag et al., 2002; Olofsson et al., 2004) or a tetramer (Enevqvist et al., 2000; Ferrão-Gonzales et al., 2000).

**Stabilization of the tetramer inhibit amyloid formation**

Independent of what building block forms the amyloid fibrils, there is clearly a connection between tetramer stabilization and inhibition of amyloidogenesis (Peterson et al., 1998; Klabunde et al., 2000; Hammarström et al., 2001a; Hammarström et al., 2002). Therefore, stabilization of the tetramer will result in a protein that is less prone to aggregate into fibrils (Miroy et al., 1996; Hammarström et al., 2002). One *in vivo* example of this is the TTR-T119M mutant which is known to be non-amyloidogenic and has the ability to suppress amyloid formation
in T119M/V30M heterozygotes (Coelho et al., 1993; Coelho et al., 1996). This stabilization was mimicked in vitro to get clues on amyloidogenicity. When one or more TTR-T119M monomer(s) was incorporated into a mixed tetramer with wild-type TTR stabilization was obtained (Hammarström et al., 2001b).

Biochemical analysis shows that the thermodynamic stability indicates whether a mutant can form fibrils or not, and the rate of tetramer dissociation (kinetic stability) decides the rate of amyloid formation and thereby the age of onset (Hammarström et al., 2002). If both the kinetic and thermodynamic stability of a mutant are known, it is therefore possible to make an estimate about severity, penetrance and age of onset of the disease (compared to other known mutants, Hammarström et al., 2002). So far, two cases disagree with this model, ATTR-D18G and ATTR-A25T, which both aggregate in the central nervous system (Hammarström et al., 2003a; Sekijima et al., 2003). These mutants have very destabilized tetramer structures with extremely fast kinetics. Their thermodynamics show abundant aggregation even at close to physiological pH, indicating that the patients should have a severe disease, with 100% penetrance and low age-of-onset. The patients get a severe disease (aggregation in the CNS) with a high penetrance, however the age-of-onset is remarkably high, as they do not exhibit symptoms until their mid-forties (Hammarström et al., 2003a; Sekijima et al., 2003). This phenomenon is believed to be due to clearance of unstable monomer by a security system in the cells, since the variant protein was found at very low concentration both in the plasma and in the cerebrospinal fluid (CSF).

The most commonly used methods to measure protein stability for transthyretin are denaturation of the protein by increasing concentration of urea or by lowering the pH (4-5.5). Evaluation of the denaturation is typically done by spectroscopic methods or by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Denaturation of TTR by urea is measured by fluorescence where a shift in wavelength from 335 to 355 is observed upon denaturation (Hammarström et al., 2001a). The denaturation curves in urea coincide almost perfectly with fraction monomer/tetramer in the sample (Hammarström et al., 2002). Fraction tetramer has been measured using a small molecule, which fluoresces when bound to at least one of the two thyroid-binding sites in the tetramer. TTR is resistant to SDS-induced denaturation unless the pH is lowered. Tetramer to monomer transition can be studied as a function of pH and measured by SDS-PAGE (McCutch en et al., 1993; McCutch en et al., 1995). Lowering pH is also used for in the turbidity assay, where amyloid aggregate formation is measured by spectroscopy (Lai et al., 1996). The pH value at the peak for amyloid formation is similar to the pH-midpoint for the tetramer to monomer transition (McCutch en et al., 1993; McCutch en et al., 1995). Generally, amyloidogenic mutants aggregate at a higher (more physiologic) pH and denature at a lower urea concentration than wild type and non-amyloidogenic mutants (Lai
et al., 1996; Hammarström et al., 2003a; Hammarström et al., 2003b; Sekijima et al., 2003; Paper II and Paper III).

**Small molecule inhibitors**

The devastating diseases caused by deposition of amyloid fibrils lead to millions of deaths worldwide. To find a cure many research groups are involved in developing inhibitors of amyloid formation. There are different approaches for inhibiting amyloid fibrillogenesis such as inhibition of interaction between GAGs and amyloid (Kisilevsky et al., 1995), removal of SAP from the blood circulation by renal clearance (Pepys et al., 2002), inhibition of the γ-secretase pathway, which leads to the production of Aβ1-42 (causing Alzheimer’s disease, Rishton et al., 2000), and inhibiting the conversion of the prion protein PrP<sup>C</sup> to the disease-causing form PrP<sup>Sc</sup> in Creutzfeldt-Jakob disease (Korth et al., 2001). In all of these approaches, small molecules (less than 1000 Da) were used. In the case of SAP the molecule, a palindromic compound based on the amino acid proline, crosslink two pentamers of SAP resulting in a decamer, which is rapidly cleared by the liver (Pepys et al., 2002). Since SAP is shown to be universally present in amyloid fibrils, it may provide a therapeutic approach to all types of amyloid. This compound is under evaluation in clinical trials.

The preservation of the transthyretin tetramer is important, small molecules that help to maintain its tetrameric form thereby serve as potential drugs for inhibition of amyloid formation. Thyroxine, the natural ligand for transthyretin, is known to inhibit amyloid formation *in vitro* (Miroy et al., 1996; Sekijima et al., 2003). Most transthyretin molecules in the brain are occupied by thyroxine and one hypothesis is that this explains why there is no amyloid formation in the human brain (Miroy et al., 1996). Since this discovery, many small molecules have been produced based on this structure using structure-based drug design (Miroy et al., 1996; Baures et al., 1998; Peterson et al., 1998; Baures et al., 1999; Klabunde et al., 2000; Petrassi et al., 2000; Oza et al., 2002). This work has resulted in powerful inhibitors that function well *in vitro*. One example is diclofenac, which has been shown to reduce amyloid formation of wild-type TTR, ATTR-Val30Met and ATTR-Leu55Pro variants in the low µM range (Baures et al., 1999; Klabunde et al., 2000; Oza et al., 2002). The low concentration needed for diclofenac makes it viable as a drug *in vivo* if the drug is metabolically stable in the body. Diclofenac is a nonsteroidal anti-inflammatory drug (NSAID) currently used for treatment of rheumatic arthritis, meaning its metabolic stability, oral absorption, and toxicity has already been proven in clinical trials. Most of the NSAIDs can, however, cause gastrointestinal ulcers that make them undesirable for treatment for chronic disorders like FAP and FAC. Studies are on-going to reduce the gastrointestinal toxicity (Oza et al., 2002).
There are, however, some examples where small inhibitors might even work in the wrong direction. T4 has been shown to induce tetramerization of the variant ATTR-D18G that is a monomer in solution, resulting in a stabilization of the mutant (Hammarström et al., 2003a). Unfortunately for patients with the Asp18Gly variant, tetramerization with the help of T4 and subsequent release of the mutated protein into the CSF is proposed to cause aggregation in the CNS causing disease (Hammarström et al., 2003a).

**Aim**

The focus of this thesis was to elucidate the mechanism of fibril formation of transthyretin and to increase our knowledge of why and how some protein molecules can change their conformation and aggregate into amyloid fibrils. This was mediated by characterizing the structures of amyloidogenic and non-amyloidogenic mutants.
Results and discussion

Structural study of transthyretin structures (Paper I)

When we started our structural study of transthyretin there were many different structures of wild types, amyloidogenic and non-amyloidogenic mutants, and complexes solved. All of these exhibited small structural differences except for the ATTR-Leu55Pro structure, which showed discrepancies in the D-strand due to the insertion of a proline in this β-strand. However, some of the, in our opinion, small alterations in the structures have been interpreted as important for amyloid formation. One limitation in the previous analyses had been the pair-wise alignment of wild type and mutant structures. To initiate our research we felt that a thorough analysis of all the 23 known structures of transthyretin was needed. The result of this analysis is described in Paper I.

As a foundation for the analysis, a new high-resolution structure of human native TTR was crucial. Therefore, the structure of a new wild-type structure was solved and refined at 1.5 Å resolution including also 43% of the data extended to 1.3 Å resolution, in space group P21212 with two monomers in the asymmetric unit. This space group is consistent with all previously solved wild-type structures and most of the amyloidogenic and non-amyloidogenic mutants.

In the new wild-type structure we observed that the N- and C-terminal ends were flexible and only the positions of amino acids 10-125 (monomer A) and 10-124 (monomer B) were well defined in the electron density. The coordinates have been deposited in the protein data bank (pdb code: 1F41, http://nist.rcsb.org/pdb/). The 1.5 Å structure is overall very similar to the previously solved wild-type transthyretin. However, the conformation of the FG-loop could be better described from the new structure. The FG-loop (amino acids Asp99-Arg103), was found and refined in two different positions in monomer B. One of the paths agrees with the previously described path in the monomer B of 1TTA (wild-type transthyretin, Hamilton et al, 1993) and the second path is similar to the one described in the A-TTRV122I mutant (1TTR; Damas et al., 1996). Due to crystal packing, both of these positions are different from the position of this loop in monomer A. The FG-loop is prone to form interactions with other residues, as in the monomer B in wild-type transthyretin. In the structure of the highly amyloidogenic mutant ATTR-L55P, all eight FG-loops form close interactions to other FG-loops from other monomers either in the same asymmetric unit or from a symmetry related molecule (Sebastião et al., 1998). The residues in the FG-loop also interact in the TTR-RBP complex (Monaco et al., 1995; Naylor and Newcomer, 1999).
Do structures of transthyretin determined at low pH represent the structure at physiological pH?

The standard crystallization protocols of transthyretin used in the past often included high salt (~2 M ammonium sulfate) in combination with a low pH conditions (pH 5.3-5.6). It has been shown that transthyretin in low pH (4.5-5.0) cause a disturbance in the tetrameric fold of the protein resulting in aggregation (Colon and Kelly, 1992; Lai et al., 1996). We therefore wanted to determine if the model at pH 5.3 represented the model at physiological conditions. Our TTR structure was obtained from a crystal grown at pH 7.0, maintaining a low salt concentration and using polyethylene glycol (PEG) as precipitant. We concluded that the changed charge distribution did not cause any large discrepancies between the reported structures and our novel wild-type structure. Therefore, the structures determined at low pH are indeed identical with their expected structures at pH 7.0, at least in the presence of high salt at the low pH levels.

No structural evidence for TTR amyloidogenesis from the 23 analyzed structures

The positioning of Cys10 has been interpreted as important for amyloid formation in the ATTR-Val30Met mutant, since analysis of ex vivo fibrils from patients carrying the ATTR-Val30Met mutation suggested that the only cysteine amino acid in transthyretin was involved in intermolecular disulphide-bond formation (Terry et al., 1993; Thylén et al., 1993). The crystal structure of ATTR-Val30Met showed a change in the position of Cys10 to a slightly more exposed position compared to the only known wild-type structure at the time (Terry et al., 1993). Comparing the structure of ATTR-Val30Met with the many structures available today showed that Cys10 is indeed generally flexible, and its conformation is not correlated to the amyloidogenic properties of the protein variant. This does not rule out the possibility that residue Cys10 is important for amyloid formation, however there is not enough structural data to support this theory. Furthermore one FAP mutation at position Cys10 (ATTR-Cys10Arg) has been identified (Uemichi et al., 1992) and it has been shown that the non-pathogenic TTR-Cys10Ala mutant can be converted to amyloid fibrils in vitro by lowering the pH (McCutch and Kelly, 1993).

In a previous analysis Schormann and co-workers found that the structures of amyloidogenic variants of TTR showed structural differences between the two monomers A and B (Schormann et al., 1998). By comparing the structures of TTR variants to wild type (pdb code: 1TTA), a high root mean square (r.m.s.) deviation was found, especially for the amyloidogenic mutants (0.4-1.0 Å superimposing the Cα atoms). In the comparison, residues Cys10-Asn123 were included. The structural differences between monomer A and B were therefore suggested to be of significance for amyloid formation (Schormann et al., 1998). From our analysis, we could show that if the residues in the flexible FG-loop (residues 99-103) are
excluded from the superpositioning, the r.m.s. deviation will be reduced to 0.1-0.5 Å. This showed that monomer A and B are close-to-identical also in the mutant structures.

Proteolytically cleaved fragments have been found in amyloid fibrils (Thylén et al., 1993). There exists a trypsin cleavage site between residues Lys48 and Thr49 (Dwulet and Benson, 1986; Wallace et al., 1986) and both of the fragments 1-48 and 49-127 have been observed in ex vivo fibrils (Thylén et al, 1993). Previously, the surface accessibility of Lys48 has been interpreted as important for amyloid formation (Schormann et al., 1998). It was proposed that Lys48 was more surface-exposed in the B monomer of amyloidogenic variants compared to B monomers of wild type and non-amyloidogenic mutants. Our analysis showed, however, that this reported increase in surface accessibility was due to the positioning of the first nine N-terminal residues of monomer B that are modeled only in some of the TTR structures. The structure of 1TTA was the best wild-type structure at the time and therefore it was used for the comparison. Unfortunately, the first nine residues of the 1TTA monomers were not well defined and were not positioned identically in the two monomers. Removal of the first nine residues prior to the surface accessibility calculation shows identical surface exposure of Lys48 in both monomers A and B. This is true for all transthyretin molecules: wild types, non-amyloidogenic or amyloidogenic mutants. This finding does not exclude the possibility that proteolytic fragments of TTR are needed for fibril formation, but points to the fact that no current structural data exists to support this hypothesis.

The temperature factor (B-factor) models the mobility of each atom in the protein. In Paper I, we compared the B-factor distribution between various structures determined at 1.9 Å or better. We found a considerable increase (3-8 Å^2) in the B-factors for the B monomers compared to the A monomers. This shows that the B monomers are generally more flexible and less well defined than the A monomers of all TTR structures. The increase in B-factors was concentrated to residues Cys10-Thr75 and residues situated in the FG-loop of each B monomer. The same effect is present in both non-amyloidogenic and amyloidogenic variants which rules out the possibility that the increased mobility in the B monomers is correlated to amyloid formation. We could not find an explanation why the B monomers are more flexible in this area.

More or less the same residues are involved in binding to ligands in the complex structures: Lys15, Leu17, Glu54, Thr106, Ala108, Leu110, Ser117, Thr119 and Val121. All these residues were well defined in the wild-type structure described in Paper I, and two (Ser117 and Thr119) are refined in two conformations. Peterson and co-workers suggested that binding of the amyloid inhibitor flufenamic acid forces residues Ser117 and Thr119 into new rotamers due to the formation of new stabilizing hydrogen bonds (Peterson et al., 1998). Our analysis showed that these rotamers are present also in structures of non-
amyloidogenic mutants as well as wild type and TTR-thyroxine complexes. Therefore, these conformations are not induced by the presence of the inhibitor.

**Recently published structures**

Since Paper I was published 20 new structures of human transthyretin have been solved. These are listed in Table 2 and a short discussion of some of these new structures will follow. In our lab, the highly amyloidogenic engineered triple mutant (TTR-G53S/E54D/L55S) was determined in the space group P3\textsubscript{2}2\textsubscript{1}1 (pdb code: 1g1o, Eneqvist et al., 2000). This structure shows a displacement of the D-strand with Leu58 in the position normally occupied by Leu55, resulting in a shorter DE-loop and an extended CD-loop. They observed a helical packing of TTR molecules with a repeat at 114.5 Å and a diameter of ~100 Å. Therefore, this packing is compatible with the structure of amyloid fibrils studied by electron microscopy and fiber diffraction (Sunde et al 1997; Eneqvist et al., 2000; Serpell et al., 2000).

A wild-type structure has been solved in, for TTR, the new space group P2\textsubscript{1}, and with two tetramers per asymmetric unit (pdb code: 1ict, Wojtczak et al., 2001). One of the tetramers was occupied with two thyroxine molecules and the other tetramer was ligand-free. This is valuable since in all former T\textsubscript{4}-complex structures of TTR the tetramer had to be generated, which results in a difficult interpretation of thyroxine binding since T\textsubscript{4} is an asymmetric molecule (Steinrauf et al., 1993; Wojtczak et al., 1996). In the P2\textsubscript{1} structure, the two thyroxine molecules had slightly different positions in each of the hydrophobic-binding pockets (Wojtczak et al., 2001). The thyroxine situated between monomers A and C is bound ~1.5 Å deeper into the channel than thyroxine in the previously described TTR-T\textsubscript{4} complex (pdb code: 2rox) but with a comparable positioning. The thyroxine situated between monomers B and D is bound even deeper and similar to 3',5'-dinitro-N-acetyl-L-thyronine (Wojtczak et al., 1996; Wojtczak et al., 2001). In the P2\textsubscript{1} structures the actual distance between the two dimers is longer for a T\textsubscript{4}-bound than for an unliganded TTR molecule (12.34 Å and 10.96 Å, respectively, between the C\textalpha atom of Ala108 and its symmetry-related equivalent across the binding site). This observation was proposed to reflect the mechanism of the ligand binding and might explain the negative cooperativity observed for T\textsubscript{4}-ligand binding.

A wild-type structure (pdb code: 1dvq) was solved by Klabunde and co-workers was used in a rational design of transthyretin amyloid inhibitors (Klabunde et al., 2000). In this study nonsteroidal anti-inflammatory drugs particularly the known inhibitor flufenamic acid were used as a starting point. Transthyretin in complex with flufenamic acid has already been structurally characterized (pdb code: 1bm7, Peterson et al., 1998). The inhibitor structures that have been studied in complex with TTR includes Resveratrol (pdb code: 1dvs), Flurbiprofen (pdb code: 1dvt), Dibenzocturan-4,6-dicarboxylic acid (pdb code: 1dvu), Diclofenac (pdb code: 1dvx), N-(meta-trifluoromethylphenyl) phenoxazine 4,6-dicarboxylic acid
Table 2 Structures of transthyretin solved since Paper I

<table>
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<tr>
<th>Structure</th>
<th>PDB</th>
<th>Year</th>
<th>Res Group</th>
<th>Space Group</th>
<th>a/b/c (Å)</th>
<th>Cryst Conditions</th>
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<th>R</th>
<th>R_{free}</th>
<th>Residues</th>
<th>#H₂O</th>
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<td>TTR G53S/E54D/L55S</td>
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(PHENOX; pdb code: 1dvy) and ortho-trifluoromethylphenyl anthranilic acid (oFLU; pdb code: 1dvz, Klabunde et al., 2000). Another inhibitor, Diflunisal, reduces amyloid formation to 17% (with no inhibitor as a reference), but it was not possible to structurally characterize this molecule in the hydrophobic pocket of TTR due to the high degree of disorder (Klabunde et al., 2000). Two molecules from this study, oFLU and PHENOX, represent the most promising candidates for a small molecule therapeutic approach against transthyretin amyloid formation. oFLU inhibited amyloid formation to 36% (with no inhibitor as a reference). It has been shown to exhibit no anti-inflammatory activity and is thereby expected to have less gastrointestinal toxicity than other NSAIDs. Gastrointestinal toxicity of NSAIDs is due to binding to an isoform of cyclooxygenase (COX-1), which ultimately result in inhibition of prostaglandin synthesis. A substituent in PHENOX was designed to be too bulky for binding to COX-1 and COX-2.

Other structures determined since our work described in Paper I was published include ATTR-Tyr114Cys (pdb codes: 1iii and 1iik, Eneqvist et al., 2002) and an engineered mutant, TTR-Phe87Met/Leu110Met, which was shown to be a monomer in solution (1gko, Jiang et al., 2001b). This mutant was crystallized in the for TTR new space group P2₁2₁2₁. Interestingly, the structure of this monomeric variant was solved from a crystal with a tetramer in the asymmetric unit, where each monomer is nearly identical to wild-type TTR.

**Flexibility of β-strand D (Paper II)**

In this work, we described an engineered double-mutant TTR-Ala108Tyr/Leu110Glu in which the mutated residues were positioned in the β-strand G with their side chains directed into the hydrophobic thyroxine-binding pocket. The relatively small hydrophobic amino acids alanine and leucine were replaced by the larger and charged amino acids tyrosine and glutamate, respectively. We expected this mutant to be monomeric and amyloidogenic. However, this mutant had the surprising effect of exhibiting more tetramer stability and less amyloidogenicity than the wild-type protein.

**TTR-A108Y/L110E is more stabile and less amyloidogenic than wild type**

We showed by urea denaturation experiments that the TTR-A108Y/L110E mutant is both kinetically and thermodynamically more stable than wild-type TTR. The midpoint for unfolding (ureaₜₐₜ) was measured to 4 M, which is 0.5 M higher than wild type (Hammarström et al., 2001a; Hammarström et al., 2002, Paper II). For the amyloidogenic mutants ATTR-V30M and ATTR-L55P much lower urea concentrations is needed to dissociate and denature the tetramer of transthyretin (ureaₜₐₜ=2-2.2 M, Hammarström et al., 2002). The mutant was also shown to have slower kinetics compared to wild type. Because of this increased stability the
mutant is less prone to aggregate into amyloid fibrils as shown by a pH-dependent turbidity assay.

**Two structures of TTR-A108Y/L110E**

The transthyretin double mutant TTR-Ala108Tyr/Leu110Glu crystallized in two different crystal forms (rod- and diamond shaped) from the same crystallization drop. The crystals belong to two different space groups, C2 and P2₁₂₁₂. We solved and characterized the high-resolution structures of the double mutant from both crystal forms. Like the previously determined structures of TTR in space group P2₁₂₁₂, contained the structure of TTR-A108Y/L110E a dimer in the asymmetric unit with a unit cell similar to wild type. The overall structure of TTR-A108Y/L110E in space group P2₁₂₁₂ (TTR-A108Y/L110Eₚ₂₁₂₁₂) was very similar to the wild-type structure with the exception of the loop region situated between β-strand B and C (amino acids 36-40) in monomer A, that has a slightly different path (Fig. 4A). This BC loop is not involved in crystal packing interactions with symmetry-related transthyretin molecules, and we found no explanation as to why this loop differs from wild type. The TTR-A108Y/L110E structure, which was determined from the C2 crystals (TTR-A108Y/L110E₁₂), had a dimer and two monomers in the asymmetric unit. TTR-A108Y/L110E₁₂ is also similar to the wild type and A108Y/L110Eₚ₂₁₂₁₂ structures, with the exception for residues 55-58 situated in β-strand D and the β-bulge region (Fig. 4B).

The increased stability of the tetramer was suggested to be due to increased interactions across the hydrophobic channel with hydrophobic interactions made by Tyr108 and the hydrogen bond between Glu110-Thr’119. The introduction of two large side-chains at the hormone-binding channel increases the dimer-dimer interface with 300-400 Å² relative to the wild-type protein. This was due to the introduction of larger side-chains at the mutation sites and because the dimers were more densely packed.

**Metastability of β-strand D**

The β-strand D is defined by three hydrogen bonds to β-strand A; one from the main-chain carbonyl oxygen of Gly53 to the main-chain nitrogen of Val16, and two from the main-chain carbonyl oxygen and amino group of Leu55 to the main chain nitrogen and carbonyl oxygen of Val14 (Blake et al., 1978; Paper I). Leu55 is also the first residue in the β-bulge that immediately follows β-strand D and comprises residues Leu55-Leu58 (see Fig. 4B). The TTR-A108T/L110E structures determined from the two different space groups deviate in this area. A large shift in the positions of Leu55 and His56 in the C2 structure breaks the two hydrogen bonds, which bridge the main-chain atoms of Leu55 and Val14. In contrast the P2₁₂₁₂ structure maintains the β-strand D. The β-strand D is absent in three out of
Figure 4. A) Main-chain (N,Cα, C) plot of human wild type (1F41, Paper I) in light grey, TTR-A108Y/L110E in blue and TTR-A108Y/L110E in red showing the differences in structure. All monomers are superimposed on monomer A from wild-type TTR. B) The β-bulge and D strand differences in TTR-A108Y/L110E in dark colors and wild type in light colors. Monomer A from the mutant is superimposed in monomer A from wild type.

four monomers in the asymmetric unit, while in monomer B it is present due to interactions with a symmetry related A’-Glu62. This A’-Glu62 form hydrogen bonds to main-chain atoms in the β-bulge, residues His56 and Gly57, due to the flipped peptide of His56. This monomer still displays a large shift of residues His56 and Gly57 compared to wild type and TTR-A108T/L110E. This shows that large conformational changes in this area can be induced by packing to neighboring residues.

Large shifts in the D-strand have otherwise only been observed in the structures for the amyloidogenic mutants ATTR-L55P and TTR-G53S/E54D/L55S (Sebastião et al., 1998; Eneqvist et al., 2000). The insertion of a proline in the D strand of ATTR-L55P disrupts the β-strand since the main-chain imino group of proline can not function as a hydrogen-bond donor, resulting in a new conformation of Pro55-Gly57 (Sebastião et al., 1998). In the TTR-G53S/E54D/L55S structure a shift of the β-strand D is observed, where Leu58 occupies the position Leu55 now mutated to serine (Eneqvist et al., 2000). Our new data show that large movement in the D-strand area can also occur in non-amyloidogenic mutants.

The structure of wild-type transthyretin has been solved in space group P2₁ at 3.0 Å resolutions (1ICT, Wojtczak et al., 2001). We observed that all except one of the eight monomers in the asymmetric unit in this crystal form are missing one hydrogen bond between the main-chain carbonyl oxygen of Leu55 and the main-
chain nitrogen atom of Val14. This suggests that β-strand D is more flexible in transthyretin molecules packed in \( \text{P}_2_1 \) crystals than TTR molecules packed in \( \text{P}_2_1 \text{,}2_1 \text{,}2_1 \). These data indicate that wild type human transthyretin has a flexible D strand, but when TTR is crystallized in space group \( \text{P}_2_1 \text{,}2_1 \text{,}2_1 \), which represents the most common crystal form, the β-strand D is intact. Other conformations of the D-strand area seem harder to crystallize, and if they do, the crystals tend to diffract at a lower resolution (Sebastião et al., 1998; Eneqvist et al., 2000; Wojtczak et al., 2001).

**Packing interactions of TTR-A108Y/L110E in space group C2**

Crystal packing of TTR-A108Y/L110E in space group \( \text{C}_2 \) involves mainly interactions between loop regions. These packing interactions include: the C strand of monomer D (residues Phe44-Lys48) packed against the DE and FG loops of monomer C and the D strand of monomer B (Ser53-Leu55) packed against the DE loop of monomer A: a hydrogen bond between the side-chain of Arg21 and the main-chain oxygen atoms of Leu82 and Gly83 from a symmetry-related monomer: and hydrogen bonds between the side-chain of Ser100 and the side-chains of symmetry-related residues Ser100, Arg103 and Asn124. The highly amyloidogenic mutant ATTR-Leu55Pro was also crystallized in space group \( \text{C}_2 \). The new crystal contacts observed in this structure were suggested to be significant for the packing arrangement in the fibril (Sebastião et al 1998). These are, however, the same contacts as observed in our \( \text{C}_2 \) structure indicating that these particular interactions are not specific for amyloid formation.

**The effect of iodide on stability and structure of transthyretin (Paper III)**

The iodide ion is one of the precursors in thyroxine biosynthesis, and until now no studies have been reported of the effect free iodide ions have on human TTR. During a crystallization setup we found that iodide improved the shape and size of the TTR crystals. Chloride ions had previously been shown to stabilize wild-type TTR (Hammarström et al., 2001a). This led us to believe that iodide also might have a stabilizing effect on transthyretin. In particular, we wanted to identify the exact binding sites for the chloride and iodide ions on TTR. We therefore characterized the iodide-bound TTR structure to 1.8 Å by the single-wavelength anomalous dispersion (SAD) method utilizing the heavy atom character of iodide. In addition, we also solved and characterized the chloride bound-TTR structure to 1.9 Å.
Iodide stabilizes the native fold of transthyretin

The protein stability in urea was determined for TTR in various salt (I or Cl) concentrations. We found that chloride stabilized TTR and for example at 1.5 M it was not possible to denature the protein even at 6 M Urea. This finding also agreed with previous published results (Hammarström et al., 2001a). For iodide, the same stabilization effect could be obtained with much lower concentration (0.1 M), thus iodide stabilized transthyretin significantly better than chloride. Interestingly, this binding is not solely due to a salting-out effect since, according to the Hofmeister series, chloride has a better salting out effect. Due to the increased stability, we observed a reduced aggregation of transthyretin by 60% at pH 4.4 in the turbidity assay and a peak shift to lower pH (pH 3.3-3.66) when 0.1 M iodide was added.

The structure of iodide-bound TTR was solved by the SAD method

A commonly used heavy atom for solving structures by SAD is selenium. However for selenium-SAD experiments a synchrotron source must be used, where the wavelength is tunable (Fig. 5). Dauter and co-workers have developed an easy method for incorporating halide ions (iodide, bromide) into the ordered solvent region surrounding protein molecules in a crystal (Dauter et al., 2000, Dauter and Dauter, 2001). A short soak (less than one min) of the crystal in a solution containing halides leads then to incorporation. The major advantage of using halides rather than selenium is that diffraction data can be collected using our in-house facility (Fig. 5)

The SAD method is based on the fact that electrons of some heavy atoms absorb x-rays of specific wavelengths. This results in detectable differences in the amplitudes for the Friedel pairs leading to a violation of Friedel’s law (which states that members of a Friedel pair have equal amplitude and opposite phase). The difference in Friedel pairs is called anomalous scattering and is used in SAD to solve the so-called “phase problem”. When the phase problem is solved, electron density maps can be calculated into which the protein structure can be built.

This method was utilized in the iodide-bound transthyretin structure, where bound iodide ions served as a heavy atom. For us this was a fast method to solve the structure due to an easily interpreted electron density map.

The structure of iodide-TTR and chloride-TTR and positioning of the ions

Overall, the refined protein structures are very similar to the 1.5 Å wild type TTR structure (Paper I) with three iodides/chlorides bound to each one of the protein monomers. Since the binding sites of the iodides and chlorides are close-to-identical, I only discuss the binding of the iodides. Halides, in contrast to metal ions, bind unspecifically to proteins and show no preferences for specific
coordination geometry. The highest occupancies for halide ions occur when ion pairs are formed with positively charged Arg or Lys side chains (Dauter et al., 2000). One of the three iodides bound to TTR is positioned at the monomer-monomer interface (Fig. 6). This iodide bridges two monomers, leading to increased stabilization of the dimer. The two remaining iodides are positioned at the T4-binding channel (Fig. 6). From the thyroxine-TTR structure, three halogen-binding pockets (HBP1-3) have been identified in the TTR monomer (Wojtczak et al., 1996). We found that the two iodide ions were positioned almost identical to the positions of the iodines in thyroxine (HBP1 and HBP3). This is surprising, since the chemical properties of charged iodide ions and the neutral iodines in thyroxine are so different. The iodide-binding sites in the halogen-binding pocket are positioned at symmetrical sites across the T4-binding channel due to 2-fold symmetry. We hope this knowledge might aid the on-going projects in structural-based drug design, which aim to produce inhibitors of amyloid formation. The physiological role of iodide binding is not clear. Iodide is found in the µM range in serum. As far as we know no studies have been made correlating FAP with iodide deficiency. It would be interesting to see if such a connection exits.
Figure 6. Binding sites of the halide atoms. The free halides are depicted in pink and included are also the positions of thyroxine in ball-and-stick (iodines from thyroxine are in cyan).
Conclusions

- Analysis of amyloidogenic and non-amyloidogenic structures shows that the structural differences between those are less than the structural difference between two independently determined wild-type structures.

- Parts of the transthyretin molecule are more metastable in solution than what has previously been recognized. This instability might, however, not lead to amyloid formation.

- The SAD method may be a simple way to elucidate the starting phases from a heavy metal-soaked protein structure. Using our in-house facility, the iodide loaded-TTR structure was easily solved based on the anomalous scattering from iodide.

- Two of the three previously identified halogen-binding pockets are also optimal for halide binding. This could prove to be a valuable contribution in the extensive efforts in designing amyloid inhibiting drugs.
Acknowledgement

Först och främst skulle jag vilja tacka min otroligt duktiga handledare Liz för att du alltid tar dig tid (som du inte har någon över) när jag behöver och för att du helt enkelt kan allt!

Fredrik E din sportfreak, du har försökt att smitta av dig med berättelser om dina jobbiga, men trevliga pass på klätterväggen, i gymmet, runt Nydalasjön, i Nydalasjön med din kajak. Men jag har lyckats motstå frestelsen att röra på mig. Tja, nästan i alla fall, jag har varit en periodare och utan dig skulle jag nog inte alls ha tränt lika mycket. Så man får tacka för peppningen och för att du varit en mycket god vän under alla dessa år! Stefan, för hjälp med programmen när de inte fungerar som de ska och för att du är en sån mästerfotograf… Erik, min gode Everquest-kumpan, för att du är så snäll och trevlig och för alla diskussioner som kanske inte alltid var jobbrelaterade. Anders K för att du är en bra kompis och en bra arbetskamrat som klarar av att hitta allt på nätet även sånt som inte finns. Ulrika för att du är så trevlig och hjälpsam och för att du var medskribent på Paper III mot ditt vetande! The lab, both past and present (Therese, Talal, Tobias, Jeanette, Christin G, Anders O, Lennart and the new guy Alex), for all the help and valuable discussions. Uwe och Shenghua for valuable help with data collection and processing of the data! Uwe you owe me a sip of your Ardbeg 1974, we should have a whisky-provning some day.

Marek, the computer guru – nothing would have worked without you och Maria sekreterare för allt pappersarbete – inget skulle ha funkat utan dig!

Linda F, Linda Ö, Caroline och Magda-Lena för trevliga pratstunder om jobb och annat.

I would also like to thank the rest of the people at UCMP for a nice working environment.

Annica, Fredrik G och Fredrik E för strategidiskussioner kring middagsbordet om hur vi ska ta oss igenom doktorandtiden och vad vi ska göra när vi blir stora!

Jag skulle också vilja tacka mitt adoptiv-lab hos Berghard-Bohms (Guggen, Viktoria, Marianne, Marie, Mattias och Björn när det begav sig) för trevliga pratstunder.

Kidde för att du är så snäll, glad och positiv och aldrig tackar nej till att umgås, vare sig det är FEST, video eller en biofilm!
Tacktack grabbgänget (Johan, Tomas, Magnus, Matts, Pär, Pontus) för alla trevliga, långa luncher, otaliga fester, frisbeegolf matcher och allting annat vi har upplevt tillsammans genom åren.

Magnus och Mirjam för alla dessa år tillsammans. Ni har varit ett underbart stöd för mig de senaste 10 eller så åren!

Kompisarna Lars J, Joar och Nilso som flydde från stan så fort de fick chansen (och jobb), jag tycker att det är dags att flytta tillbaka snart!

Tack till min underbara familj Mamma och Lars, Pappa och Ewa, Annette, Erica och Claes och Johanna för att ni alltid finns omkring mig!!!

Sist, men absolut inte minst, skulle jag vilja tacka MARIA (även kallad Älsklingsärleksbäjbelovven) för allt stöd och all hjälp och för att du är den bästa flickvän man kan tänka sig! Jag älskar dig!
References


