Molecular Aspects of Transthyretin Amyloid Disease

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Att springa vilse ibland gör att man får upptäcka mer av världen
Abstract

This thesis was made to get a deeper understanding of how chaperones interact with unstable, aggregation prone, misfolded proteins involved in human disease. Over the last two decades, there has been much focus on misfolding diseases within the fields of biochemistry and molecular biotechnology research. It has become obvious that proteins that misfold (as a consequence of a mutation or outer factors), are the cause of many diseases. Molecular chaperones are proteins that have been defined as agents that help other proteins to fold correctly and to prevent aggregation. Their role in the misfolding disease process has been the subject for this thesis.

Transthyretin (TTR) is a protein found in human plasma and in cerebrospinal fluid. It works as a transport protein, transporting thyroxin and holo-retinol binding protein. The structure of TTR consists of four identical subunits connected through hydrogen bonds and hydrophobic interactions. Over 100 point mutations in the TTR gene are associated with amyloidosis often involving peripheral neurodegeneration (familial amyloidotic polyneuropathy (FAP)). Amyloidosis represents a group of diseases leading to extra cellular deposition of fibrillar protein known as amyloid. We used human SH-SY5Y neuroblastoma cells as a model for neurodegeneration. Various conformers of TTR were incubated with the cells for different amounts of time. The experiments showed that early prefibrillar oligomers of TTR induced apoptosis when neuroblastoma cells were exposed to these species whereas mature fibrils were not cytotoxic. We also found increased expression of the molecular chaperone BiP in cells challenged with TTR oligomers.

Point mutations destabilize TTR and result in monomers that are unstable and prone to aggregate. TTR D18G is naturally occurring and the most destabilized TTR mutant found to date. It leads to central nervous system (CNS) amyloidosis. The CNS phenotype is rare for TTR amyloid disease. Most proteins associated with amyloid disease are secreted proteins and secreted proteins must pass the quality control check within the endoplasmic reticulum (ER). BiP is a Hsp70 molecular chaperone situated in the ER. BiP is one of the most important components of the quality control system in the cell. We have used TTR D18G as a model for understanding how an extremely aggregation prone protein is handled by BiP. We have shown that BiP can selectively capture TTR D18G during co-expression in both *E. coli* and during over expression in human 293T cells and collects the mutant in oligomeric states. We have also shown that degradation of TTR D18G in human 293T cells occurs slower in presence of BiP, that BiP is present in amyloid deposition in human brain and mitigates cytotoxicity of TTR D18G oligomers.
Included papers

Paper I:

Paper II:
Prefibrillar Amyloid Aggregates and Cold Shocked Tetrameric Wild Type Transthyretin are Cytotoxic. Sörgjerd K, Klingstedt T, Lindgren M, Kågedal K, Hammarström P. In manuscript.

Paper III:

Paper IV:
BiP can function as a molecular shepherd that alleviates oligomer toxicity and amass amyloid. Sörgjerd K.,Wiseman R.L, Kågedal K., Berg I., Klingstedt T., Budka H, Nilsson K.P.R., Ron D., Hammarström P. In manuscript.
**Sammanfattning**

Denna avhandling handlar om proteiner. Särskilt de som inte fungerar som de ska utan har blivit vad man kallar ”felveckade”. Anledningen till att proteiner veckas fel beror ofta (men inte alltid) på mutationer i arvsmassan. Felveckade proteiner kan leda till sjukdomar hos människor och djur (man brukar tala om amyloidsjukdomar), ofta av neurologisk karaktär. Exempel på amyloidsjukdomar är polyneuropati, där perifera nervsystemet är drabbat, vilket leder till begränsad rörelseförmåga och senare till förlamning; och Alzheimer’s sjukdom, där centrala nervsystemet är drabbat och leder till begränsad tankeförmåga och minnesförluster.

Studierna som presenteras i denna avhandling har gått ut på att få en bättre förståelse för hur felveckade proteiner interagerar med det som vi har naturligt i cellerna och som fungerar som skyddande, hjälpande proteiner, så kallade chaperoner.

Transtyretin (TTR) är ett protein som cirkulerar i blodet och transporterar tyroxin (som är ett hormon som bland annat har betydelse för ämnesomsättningen) samt retinol-bindande protein (vitamin A). I TTR genen har man funnit över 100 punktmutationer, vilka har kopplats samman med amyloidsjukdomar, bland annat ”Skellefteåsjukan”. Mutationer i TTR genen leder ofta till att proteinet blir instabilt vilket leder till upplösning av TTR tetrameren till monomerer. Dessa monomerer kan därefter sammanfogas på nytt men denna gång på ett sätt som är farligt för organismen. I denna avhandling har fokus legat på en mutation som kallas TTR D18G, vilken har identifierats i olika delar av världen och leder till en dödlig form av amyloidos i centrala nervsystemet.

Det chaperon som vi har studerat benämns BiP och är beläget i en cellkomponent som kallas för det endoplasmatiska retiklet (ER). I ER finns cellens kontrolsysteem i vilket det ses till att felveckade proteiner inte släpps ut utan istället bryts ned.

Denna avhandling har visat att BiP kan fånga upp TTR D18G inuti celler och där samla mutanten i lösliga partiklar som i detta fall är ofarliga för cellen. Avhandlingen har också visat att nedbrytningen av TTR D18G sker mycket långsammare när BiP finns i riklig mängd.
### Abbreviations

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<tr>
<td>ANS</td>
<td>8-anilino-1-naphthalene sulfonic acid</td>
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<tr>
<td>Bis-ANS</td>
<td>4-4-bis-1-phenylamino-8-naphthalene sulfonate</td>
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<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>CtD</td>
<td>C-terminal domain</td>
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<tr>
<td>DCVJ</td>
<td>4-(dicyanovinyl)-julolidine</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>FAP</td>
<td>familial amyloidotic polyneuropathy</td>
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<tr>
<td>LCP</td>
<td>luminescent conjugated polymer</td>
</tr>
<tr>
<td>MTT</td>
<td>3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NBC</td>
<td>neuroblastoma cells</td>
</tr>
<tr>
<td>NtD</td>
<td>N-terminal domain</td>
</tr>
<tr>
<td>RBP</td>
<td>retinol binding protein</td>
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<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SSA</td>
<td>senile systemic amyloidosis</td>
</tr>
<tr>
<td>ThT</td>
<td>thioflavin T</td>
</tr>
<tr>
<td>T4</td>
<td>thyroxin</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
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<tr>
<td>TTR</td>
<td>transthyretin</td>
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<tr>
<td>TTR D18G</td>
<td>transthyretin with amino acid substitution from aspartic acid to glycine at position 18</td>
</tr>
<tr>
<td>UPR</td>
<td>unfolded protein response</td>
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Tack


De jag vill tacka är:


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BiP can protect cells from TTR D18G cytotoxicity
BiP is found in TTR D18G aggregates in patient tissue

Conclusions

References
Introduction

This thesis summarizes what I have been working on for the past five years and what conclusions I have made from my findings. My main characters are two proteins called BiP; which is a molecular chaperone believed to play a protective role in cells, and transthyretin (TTR); which is associated with human misfolding disease. It has been known for a long time that TTR misfolding disease starts with TTR denaturation and leads to aggregation and fibrillation of TTR, which accumulates in tissues and organs in patients suffering from the disease. Still, there are no cures for most of these kinds of diseases and the pathogenesis and mechanisms are not fully understood.

The aims with my studies have been to elucidate what role BiP plays in TTR misfolding diseases. I have specifically studied a mutant of TTR called TTR D18G, since that mutant is the most destabilized and most unusual form of TTR. I have also aimed to follow the mechanisms for TTR misfolding and to study the consequences in human cells when exposed to misfolded variants of TTR.

I have included four papers in this thesis. In the first, the aggregation process of transthyretin is described, and the different states in the process are characterized. In the second paper, the effect of different conformational states of aggregated TTR variants on human cells has been studied. In the third paper, the interactions of TTR D18G with BiP are characterized and hypothesizes about what role BiP plays in TTR misfolding diseases have been made. In the fourth paper, the BiP TTR D18G interaction is studied from a mammalian point of view and the effects of BiP and TTR D18G on human cells are elucidated.
Proteins

Proteins are responsible for most of the reactions occurring in the human body such as transport of nutrients and oxygen, defense against microorganisms, control of gene expression, transmission of signals etc. In all organisms and in each cell, they exist and they work. In the human body, over 30,000 different proteins (and around 30,000 protein coding genes) [1] with almost as many different functions are present, however, most of them still have unknown functions. The building blocks for proteins are called amino acids. There are 20 amino acids used for protein synthesis and 12 of them can be produced by human cells whereas eight need to be supplied with the diet. Thus, a versatile diet is important for the body to work properly with all its > 30,000 different proteins to be properly synthesized. An average protein consists of hundreds of amino acids, linked together in different sequences by the peptide bond, and it is the order of the amino acids that dictate the final shape of the protein. The amino acids, the building blocks in a polypeptide, have different properties; they can be polar, non-polar or charged and the hydrophobic ones are usually buried in the interior of the folded protein. The structures of the proteins, i.e., their conformations, differ due to different types of secondary structures, called α-helices or β-sheet and how these structural elements are arranged. It is the conformation that dictates the protein function. Every single amino acid in the folded protein can contribute to and play a role for protein function. A substitution of one amino acid to another might in some cases lead to re-arrangements of the whole protein structure, and thereby induce a new behavior of the protein (often leading to destabilization and degradation). In other cases, an amino acid substitution does not influence the conformation at all.
Figure 1) The primary structure of a protein showing amino acids as a string of pearls. The side chains of the amino acids, denoted with R can be polar, non-polar or charged. When the amino acids are connected to form a poly peptide chain, the COO$^-$ group of one amino acid reacts with the NH$_3^+$ of another, and a peptide bond is formed with release of a water molecule.

Protein molecules can interact with each other, and protein-protein interactions are necessary for many biological functions. Interactions can be prolonged, when a complex is formed, or transient, i.e. when signals are transferred within or between cells. Interactions can also be non-preferred, such as protein aggregation.

Protein production- the background story

All proteins begin as linear sequences of amino acids linked together as a string of pearls (Figure 1). The information about the amino acid sequence of the protein, leading to their different conformations is encoded in the deoxyribonucleic acid (DNA), in the specific genes for the proteins of interest, called the genetic code. When synthesis of a polypeptide begins, the DNA information is transferred to a piece of messenger RNA (mRNA). Formation of RNA from DNA is a process called transcription and occurs with help from an enzyme called RNA polymerase. DNA was identified in 1944 and its double helical conformation was revealed in 1953 [2]. Since DNA is situated in the cell nucleus whereas the protein synthesis occurs in the cytoplasm, an intermediate needs to be involved in the transcription. In the fifties,
there were discussions about that intermediate and it was proposed that another nucleic acid, single stranded ribonucleic acid (RNA), would be the intermediate responsible for transferring information from the cell nucleus to the cytoplasm. Later, it was formulated that the genetic information in the DNA is transcribed to RNA and then translated from RNA into a protein. The idea was developed over time and in the sixties, it was proposed that a gene is transcribed into a specific RNA species, called mRNA and that a short-lived, non-ribosomal RNA directs the synthesis of proteins. In 1965, Francois Jacob, Jacques Monod and André Lwoff received the Nobel Prize for their research about mRNA. In the seventies, it became known that mRNA could be spliced after transcription, resulting in that the primary transcript can generate different mRNAs and different proteins. In the 1980s, it was found that small RNA molecules could bind to a complementary sequence in mRNA and inhibit translation [3]. mRNA is single stranded and its sequence is called sense because it results in a protein. The complementary sequence is called antisense. When sense mRNA base pair with antisense mRNA, translation is blocked. The mechanism has not been fully understood until Craig C. Mello coined the term RNA interference in his work from 1997, published in Cell [4]. In 2006, Andrew Z. Fire and Craig C. Mello received the Nobel Prize for uncovering the mechanism of RNA interference. They discovered that genes could be silenced, i.e. gene activity could be turned off, by double-stranded RNA [5].

Protein production is initiated by transcription from DNA to mRNA. The transcription starts with binding of RNA polymerase to the DNA which, together with different cofactors, unwinds the DNA. The unwinding helps the RNA polymerase to bind the single stranded DNA template. But there is also need for different transcription factors to make the interaction possible. Once RNA polymerase has bound, the elongation starts, which means that an RNA copy of the DNA template is made as RNA polymerase is traversing along the template strand. The copy (mRNA) is transported to the cytoplasm once it is finished. In the cytoplasm, the sequence is translated into amino acids with help from the ribosome. Ribosomes consist of different subunits that surrounds mRNA and use its sequence as a template for amino acid synthesis where the ribosome is constantly fed with amino acids from transport RNA (tRNA) molecules, each specific for one amino acid (Figure 2). When the amino
From DNA to protein.

In the nucleus, mRNA is made, which is a copy of the DNA template containing all the genetic information. Protein synthesis is performed in the cytoplasm by the ribosomes.

Once the amino acid sequence is finished, it is released from the ribosome and folds into a three-dimensional structure and is transported to its predestined destination.

**Protein folding**

The normal protein function does not appear until the polypeptide has developed its final three-dimensional conformation, *i.e.* the protein has been folded. Protein folding involves interactions of the amino acids within the polypeptide to form different kinds of secondary structures; α-helices and β-sheet (Figure 3). The secondary structure elements can arrange into a tertiary structure mediated by side chain interactions. The folding process occurs right after the synthesis of the polypeptide and is normally a relatively fast process, it can even occur on a milli or micro second time scale. The
American biochemist Christian Anfinsen was the first to show that the order of amino acids in the primary structure is what dictates the final protein conformation [6]. He also found that if a folded protein was denatured, \emph{i.e.} the non-covalent native H-bonds, the charge-charge interactions and the hydrophobic interactions were broken and the protein became unfolded, it could again find its folded, native conformation, \emph{i.e.} refold, under permissive conditions. Anfinsen was awarded the Nobel Prize in 1972. However, for many proteins, the process is not as simple as it was initially described by Anfinsen. Some proteins can fold, unfold and refold spontaneously \emph{in vitro} (usually the smallest ones) in a one-step process [7], some fold through one or many intermediates and most need assistance to adopt their ultimate conformation. The cells are provided with molecules whose major function is to help proteins to fold correctly, these molecules are also proteins, and are called molecular chaperones.

\textbf{Genetic mutations}

Mutations are permanent alterations in the DNA sequence of an organism and are classified as point mutations (often leading to amino acid substitutions), insertions (where a nucleotide has been added to the DNA sequence) or deletions (where a nucleotide has been removed from the DNA sequence). There can also be large scale mutations in the chromosomal structure leading to loss of genes or massive repetitive insertions of DNA. Most mutations do not have any significantly effect on the organism, but some of them are harmful. Mutations occur in two different ways; they are either inherited, \emph{i.e.} they are passed from parent to child (and are present in every cell throughout the person’s life), or they are acquired during the lifetime. Acquired mutations are usually results of environmental factors e.g. exposure of DNA to UV light, viral infections, chemicals etc. But they can also be copying errors during division of cells. Acquired mutations cannot be passed on to the next generation unless they have occurred in sperm or egg cells.
Protein misfolding

When errors occur in the protein folding machinery, it can result in protein misfolding and misfolding disease. Misfolding diseases are often associated with amyloidosis [8]. The reasons for protein misfolding could be mutations in the genes that code for the proteins that are to be misfolded, or outer factors like stress. For some cases, it is unknown why proteins start to misfold. The consequences could be harmful to the surrounding cells and to the organism in general. Protein misfolding diseases strike many people during their lifetime, and it seems like the phenomenon has become more prevalent during the past years. One early disease of this kind was described in 1906, when the German neurologist Alois Alzheimer found a form of amyloidosis that affects the brain. The disease was later named Alzheimer’s disease (AD). Most AD patients get the disease sporadically, i.e., it is usually not inherited. The symptoms for disease include memory disturbance and loss of other intellectual abilities, the symptoms are also called dementia. To date, more than 20 million people are believed to suffer from dementia [9]. In the fifties, the first form of transmissible human amyloid disease, named kuru, was found among people practicing cannibalism in Papua New Guinea. Shortly afterwards, a disease with similar pathology was discovered in Europe and United States; transmissible spongiform encephalopathy (TSE), among individuals that had been treated with growth hormones extracted from human cadavers. The TSEs include Bovine Spongiform Encephalopathy or Mad Cow Disease.
Disease (BSE), Creutzfeldt Jakob Disease (vCJD), Gerstmann-Straussler-Scheinker (GSS) disease and fatal familial insomnia (FFI). Certain misfolded proteins, called prions, are implicated in these diseases. The word "prion" stands for "proteinaceous infectious particle", referring to its pathogenic variants. Stanley B. Pruisiner was the first to identify the molecular mechanisms of prions [10-13] and he was awarded the Nobel Prize in Medicine in 1997 for discovering a new infectious agent-a protein.

All misfolding diseases, whether they are sporadic, inherited or transmissible, are associated with deposition of proteins in different organs, depending on the disease (Table 1). The proteins involved are normally soluble but have become insoluble and aggregated and have developed fibril-like structures. Remarkably, the final fibrils are strikingly similar regardless of the precursor protein, consisting of cross-β-sheet structure with twisted morphology. The mechanisms for developing fibrils are also similar; starting with a conformational change in the protein which becomes a building block for aggregates or clusters that develop long fibril-like structures over time and accumulate in tissues or organs in the body with consequences like impaired organ function and cell death [14].

Alzheimer’s, Parkinson’s and Creutzfeldt Jakob disease are examples of notorious misfolding diseases, but there are also less known diseases like Familiar amyloidotic polyneuropathy (FAP) with related pathology (Table 1). The precursor protein for FAP is transthyretin (TTR).
<table>
<thead>
<tr>
<th>Clinical syndrome</th>
<th>Precursor protein</th>
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<tr>
<td>Alzheimer's disease</td>
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<td>Senile Systemic Amyloidosis (SSA)</td>
<td>Transthyretin</td>
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<tr>
<td>Familial Amyloid Polyneuropathy (FAP)</td>
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<td>Type II Diabetes</td>
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<td>Familial Amyloidosis Type III</td>
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<td>Hereditary Renal Amyloidosis</td>
<td>Fibrinogen</td>
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**Table 1)** A selection of diseases coupled to protein misfolding and amyloidosis and their precursor proteins.
Transthyretin (TTR)

Transthyretin (TTR) was discovered in 1942, it became known as prealbumin and was by then detected in the cerebrospinal fluid. In the fifties, prealbumin was identified as a thyroxine (T4) binding protein by Sidney Harold Ingbard, which was published in 1958 in Endocrinology [15]. Kanai et al [16] characterized prealbumin as a retinol binding protein and published their finding in a paper in Journal of Molecular Biology, and prealbumin became Retinol Binding Prealbumin (RBPA). The structure of RBPA was described by Blake and colleagues in 1978 [17] and in 1981, the name transthyretin was accepted [18].

TTR has a molecular weight of 55 kDa and its structure (studied by X-ray crystallography) is a homotetramer with four identical, monomeric subunits, composed of 127 amino acids [19]. Each subunit has a molecular weight of 14 kDa and contains eight β-strands denoted A-H and a helix between strands E and F. The β-strands in each monomer form a β-barrel consisting of two antiparallel four stranded β-sheets containing the DAGH and CBEF strands. Association of two monomers to a dimer forms a β-sandwich stabilized by hydrogen bonds between the H-H (Figure 4) and F-F strands. Association of another dimeric β-sandwich produces the tetrameric conformation. The dimers are connected through hydrophobic interactions between the A-B loop of one monomer and the H-strand of the opposite dimer.
Figure 4) The dimeric form of TTR. The dimer is held together and stabilized by hydrogen bonds between strands H and H and F and F (not shown) in the TTR structure (www.pdb.org, pdb code 1DVQ).

Tetrameric TTR contains two identical T4-binding sites (Figure 5) located in a channel at the center of the molecule [20]. If one of the binding sites is occupied with T4, it becomes harder for the second T4-molecule to bind because of an allosteric effect (negative cooperativity) that takes place in the molecule upon binding of T4. T4 is a thyroid hormone and it plays a role in the metabolism, but is also important for neuronal development [21]. Free T4 is metabolically active. Plasma TTR functions as a transporter of T4 in the blood and transports 15-20% of serum-T4 and around 80% of CNS-T4 [22]. Other T4 binding proteins and transporters include albumin and thyroxine-binding globulin. TTR is also involved in the transportation of retinol (vitamin A) in complex with retinol binding protein (RBP). The TTR–RBP–retinol complex is formed in the endoplasmic reticulum (ER) of hepatocytes. In TTR, there are four binding sites for RBP, however, only two molecules can bind at the same time because of steric hindrance. In the plasma, most of the TTR does not bind RBP [23, 24]. The source for plasma TTR is the liver. In human plasma, TTR is present at
a concentration of 0.25 g/l [23]. The major sites for TTR production are in the liver, the choroid plexus of the brain and the retinal pigment epithelium in the eye.

There are over 100 known mutations in the TTR gene that are associated with amyloid deposition, with varying phenotype depending on the mutation [25] (some are shown in Figure 8). The most common neurodegenerative disease associated with TTR mutations is familial amyloidotic polyneuropathy (FAP) [23, 26-28], which first was described in 1952 by Corino Andrade [29] in Portugal. For more than 20 years, Andrade and colleagues had observed 74 cases from different families suffering from a progressive and mortal, by then unknown disease. The genetic defect and cause for disease has been identified as the V30M TTR mutation [30]. FAP V30M TTR has also been reported in Japan, Spain, America and Scandinavia [23, 31, 32] and is caused by misfolding of TTR resulting in amyloid fibril formation where TTR monomers have associated into cross-β-sheets [33]. The amyloids in FAP affect the peripheral- and the autonomic nervous system. In FAP patients, amyloidogenic, mutated TTR is produced and found in the plasma and deposited in tissues. The frequency of the mutant gene is considered at 1 in 100 000 to 1 million [34], however, some carriers never develop the disease. In patients who develop disease, the age of onset is between 17 and 78 (with more than 80% developing symptoms before the age of 40) for the Portuguese patients and it becomes fatal around 10 years after the initial symptoms [26, 35]. In Sweden is FAP known as “Skellefteåsjukan”. For Swedish patients, FAP onset is signification later in life with an average age of 57 years. The first symptoms are usually loss of sensibility in fingers and toes and walking disability. Later symptoms include cardiac dysfunction, emaciation and renal failure [35]. Since the main source of TTR production is the liver, FAP patients can be treated with liver transplantation whereby the FAP mutant secreting liver is replaced by a liver that only secretes TTR wt. Liver transplantation usually halts the disease progression and results in amyloid clearance over time, but often with cardiac amyloid progression [25].
Although TTR amyloid deposit disease is associated with TTR variants, senile systemic amyloidosis (SSA) is a disease associated with TTR wild type and affects up to 25% of people over the age of 80 and is characterized by amyloid deposits in the heart [36, 37]. The primary structure of TTR is therefore not the only explanation for development of TTR amyloidosis [38]. SSA is usually benign and without symptoms, and mostly men are severely affected [39]. Analysis of the amyloid fibril deposits in SSA patients have revealed that the amyloids contain fragments of TTR and those fragments dominate over full length TTR [40, 41]. The fragmentation has occurred at certain positions (predominantly at positions 46, 49 and 52) in the molecule which makes it tempting to believe that the cleavage of TTR is the cause of disease since the cleavage might expose sequences that are prone to aggregate. However, the cleavage mechanism is not fully understood.
Formation of TTR amyloids starts with dissociation of the tetramer into monomers, that in turn partly unfold and develop aggregates and amyloid fibrils over time [42-44] (Figure 6). While the structure and properties of amyloid fibrils have been in the focus for diagnosing and understanding the pathogenesis for amyloid disease, there is now increasing evidence that the intermediate states in the amyloid formation pathway, are the most toxic species [45-48].

Another role for TTR, which recently has been published, is the ability to bind the Aβ-protein, which plays the major role in the pathology of Alzheimer’s disease. TTR can act in a chaperone like manner and thereby prevent formation of Aβ amyloid aggregates and thereby possibly halt progression of Alzheimer disease [49].
Figure 6.) The amyloid formation process of TTR. The native tetrameric structure of TTR is destabilized and form a rearranged structure that dissociates into monomers. The monomeric species are unstable and aggregation prone and can mature into long, inert fibril structures or unfold. Unfolded TTR can also rearrange to a molten globule like structure (A-state) that has very similar properties as the monomeric amyloidogenic intermediates.

The TTR D18G mutation

TTR D18G is a naturally occurring mutation in the TTR gene. The mutation was originally discovered in a Hungarian family, where four definite and three probable affected members were identified. It leads to amyloidosis in the central nervous system (CNS) with disease onset at an average age of 44. The affected family members had extensive amyloid deposition in the leptomeningeal vessels and in the subarachnoid membrane. In all patients, the symptoms of the disease included memory disturbance, psycmotor decelleration, ataxia, hearing loss and a majority also suffered from migraine-like headache, nausea and tremor [50, 51]. The CNS phenotype is very rare for TTR disease and the fairly late age of onset is surprising since TTR D18G was identified as the most destabilized TTR mutant found to date. Recently, it has been demonstrated that a combination of thermodynamic and kinetic stability of TTR mutants is strongly correlated to disease progression. Therefore, it
was expected that the disease onset for D18G carriers should be lower. For comparison, the L55P mutation is a very aggressive FAP variant with early disease onset (patients get their first symptoms in their teens to early twenties). In L55P patients, the variant protein can be detected in serum at amounts comparable to wt subunits [52]. Analysis of serum and cerebrospinal fluid (CSF) of a heterozygote D18G patient revealed that only TTR wt could be detected [53], which is an indication of degradation or accumulation of D18G within the cell or rapid degradation post secretion. This could explain why patients do not develop disease until 44 years of age.

TTR D18G is monomeric (Figure 7) and unable to form tetramers under physiological conditions. The mutant is aggregation prone and aggregates 1000-fold faster that TTR wt under physiological conditions [53]. The location of the D18G mutation is at the end of the A-strand of TTR. The neighboring residues (Figure 7) are known to stabilize the tetrameric structure. For example, the A25T mutation results in destabilization of the TTR tetramer and cause CNS amyloidosis [54], the V20I mutation leads to destabilization of the TTR tetramer and cause cardiac amyloidosis [55], the F87M/L110M mutations engineer the TTR molecule to be monomeric [56] and the L111M mutation leads to cardiac amyloidosis [57].

**Figure 7**) Position of D18 in the TTR monomer and residues believed to influence the tetramer stability. A) Structure of a TTR monomer. Residues within a radius of B) 5Å C) 7Å
Figure 8) Primary sequence of TTR with naturally found mutations marked below the wild type sequence (Hou et al 2007).

The region with the D18 mutation obviously has high impact on TTR tetramerization and stability. TTR D18G cannot efficiently form hybrid tetramers with TTR wt. T4 binding was found to facilitate tetramerization of D18G in the choroid plexus, where concentrations of T4 is high, but not in the CSF where the concentrations are lower. That could explain the TTR D18G prevalence to accumulate in the CNS. Expression of TTR D18G in E.coli leads to the formation of inclusion bodies [53].
**Molecular chaperones**

The term “molecular chaperone” was coined by Ron Laskey in 1978. Laskey observed that a nuclear protein, called nucleoplasmin, could solve a misassembly problem during the assembly of histone proteins, termed nucleosomes, in amphibian eggs. Nucleosomes bind DNA by electrostatic interactions. If the interactions are broken, e.g. by changes in the physiological conditions, the nucleoplasmin is not able to rebind the DNA, even if the physiological conditions are readapted, which leads to aggregation of the protein. This can be prevented by the presence of nucleoplasmin, is able to bind the nucleosomes and protect them [58].

The molecular chaperones are today known as folding helpers and it is believed that they are essential for cell survival and for life processes in general. They are present in the mitochondria, the Golgi, the ER and in the cytoplasm of all cells. The chaperones can correct mistakes in the folding machinery, unfold and send misfolded species to be degraded, or hold on to proteins that cannot be folded in a productive way, thereby preventing escapes of misfolded proteins that could cause damage. The chaperones direct their substrates into productive folding, transport or degradation pathways, but they do not become parts of the final structures of the proteins they interact with [59]. The majority of newly synthesized proteins need assistance to adopt their final conformation. Molecular chaperones stabilize non-native proteins, unfold incorrectly folded proteins and send abnormal proteins for degradation. They do not interact with native proteins, only the unfolded or partially unfolded ones. They are interacting with the proteins for a finite time and thereafter release their substrates, often mediated through ATP hydrolysis. Some chaperones interact with a wide variety of polypeptide chains whereas others are very restrictive and only bind to specific targets.
The heat shock response was first discovered in 1962 in Drosophila flies and the heat shock proteins (HSP) were identified as a set of proteins whose expression was induced when the cells were exposed to elevated temperature [60]. Shortly after they had been discovered, it became evident that their synthesis was not only due to elevated temperatures in cells but also to other forms of outer stresses, like radiation (UV or gamma-irradiation), oxidative stress, exposure to heavy metals, amino acid analogues etc. Protein misfolding and aggregation can lead to acute or chronic stress and activation of inappropriate signaling pathways. HSPs have strong cytoprotective effects [61] and are thought to restore the cellular homeostasis when it is disturbed.

Mammalian HSPs are classified according to their molecular weights (in kilodaltons) and are divided into two main groups, the high molecular weight HSPs and the small molecular weight HSPs. The first group includes three major families: Hsp60, Hsp70 and Hsp90. The first group (the heavy HSPs) consists of ATP dependent chaperones whereas the second group (the light HSPs) consists of ATP independent chaperones.

The Hsp70 family is the most studied HSP family, containing proteins from 66 to 78 kDa. Some of the Hsp70 proteins are localized in the cytosol (Hsp70 and Hsp72), one is found in the mitochondrion (mtHsp70) and one in the ER (BiP).

**BiP**

BiP was first defined as glucose regulated protein with a molecular weight of 78 kDa (GRP78) or immunoglobulin heavy chain binding protein. Its function as a molecular chaperone was established by Munro et al [62] in 1986, who demonstrated that BiP is an ATP dependent member of the Hsp70 family, located in the ER lumen. BiP interacts with newly synthesized proteins and chaperones them during transport through the cell and is believed to be one of the most important components in facilitating folding in the ER. BiP has a molecular mass of 74 kDa and its 3D structure is not known but it has been defined by X-ray crystallography for DnaK (an *E.coli* Hsp70 and BiP homologue).
Structure and mechanism of BiP

All the Hsp70 family members have the same structural organization with a 44 kDa N-terminal ATPase domain (NtD), a 18 kDa C-terminal substate binding domain (CtD) (Figure 9) and a third domain, belonging to the C-terminal domain whose function is unknown. The NtD and the CtD communicate allosterically with each other. If the NtD is occupied by an ATP molecule, the affinity for the substrate in the CtD is low but if the NtD is occupied by an ADP molecule, the affinity for substrate is high. If the CtD binding site is occupied by a substrate, the rate of ATP hydrolysis in the NtD increases [63, 64]. Thus, an unfolded polypeptide captured by BiP, can undergo cycles of binding and release, cycles that will proceed until BiP binding motifs no longer are present in the released and folded polypeptide. BiP recognizes a wide variety of nascent polypeptides with no obvious sequence similarity. However, experiments that have been done in order to find sequences that BiP preferentially binds to, have shown that the binding motifs consist of a high proportion of hydrophobic residues, normally located in the interior of a folded protein. It has also been shown that those motifs preferably consist of seven amino acids [63].

Figure 9) The NtD and the CtD of the bacterial BiP homologue DnaK. The NtD binds ATP/ADP (ADP is marked with black, left figure) whereas the CtD is substrate binding (substrate (NRLLLTG) is marked with black, right figure). The domains communicate allosterically with each other. When ATP is bound to the NtD, the CtD releases its substrate (www.pdb.org, pdb code 1S3X for left figure and 1Q5L for right figure).
A computer scoring system was used to predict BiP binding sites in natural proteins [65]. The BiP score program uses an algorithm that scores the amino acid sequences for every chosen substrate, giving a measure for the binding probability of every heptapeptide. Sylvie Blond, Chicago, USA, used an updated matrix (7pep3) that includes results from initial affinity panning experiments as well as in vitro binding studies of synthetic peptides [66-69] and data provided by Dr. Mary-Jane Gething, University of Melbourne, Australia. The program calculates an integer for each heptapeptide in the substrate. A relative high value of the integer, i.e. values higher than five, means that there is a big chance that BiP would bind to the corresponding amino acid sequence. Aberrant high values for isolated heptapeptides with a proline residue at position 6 which are not flanked by a hydrophobic residue such as Phe or Trp were recalculated using the score value 0 instead of 12 for the proline contribution. In addition, the software allows prediction of putative binding sites for the bacterial DnaK based on the matrix developed by Bukau and colleagues [70] and also calculates the hydropathy index for every overlapping seven residue-long stretch of protein sequence using the hydropathy scale of Kyte and Doolittle [71]. The BiP scoring software is protected under a license agreement with the University of Illinois at Chicago and is available upon request (blond@uic.edu).

BiP can self associate into different oligomeric species and it is the Ctd that is responsible for oligomerization. The more oligomeric BiP is, the less active is it [65]. BiP can also be post-translationally modified by phosphorylation and by ADP ribosylation. These modifications are believed to play a role in the synthesis and the polypeptide binding of BiP. Accumulation of unfolded proteins in the ER leads to an decreased amount of modified BiP whereas unmodified, monomeric BiP increases [64].

Many chaperones need co-chaperones to be effective. Hsp70 chaperones often need J-domain containing Hsp40 proteins. The function for the Hsp40 proteins is to stimulate the ATPase activity which is crucial for Hsp70 chaperone activity. BiP can interact with different J-domain proteins [72] which are necessary for the chaperone function. The bacterial homologue for Hsp70 is called DnaK and the bacterial homologue for Hsp40 is called DnaJ. Interaction of DnaK with DnaJ is mediated by the J-domain of
DnaJ, which includes residues 2–75. DnaJ can function as a chaperone either by itself or in complex with DnaK [73].

**A role for BiP during translocation**

Another role for BiP besides acting as a chaperone is to block the passage of proteins into the ER, which is also believed to be a protective mechanism for cells. On the ER membrane, there are sites called translocons. A translocon functions as a passage for proteins that are going to pass the ER membrane and contains an aqueous pore, through which proteins can pass. Both on the outer side of the ER and on the ER luminal sides, there are seals that prevent small molecules to pass the ER membrane. The ER luminal seal is closed until a nascent polypeptide reaches a size of ~ 70 amino acids [74]. There are different possibilities regarding how the sealing mechanism on the luminal side works, and one possibility is that there are proteins in the ER lumen that can bind to the end of the pore and close it. Art Johnson and colleagues [75] have demonstrated that BiP seals the luminal end of the translocon to separate the ER from the cytosol and prevent small peptides to enter the ER. In other words, BiP has multiple roles in the ER and it can act as a seal only in presence of ATP/ADP.

**The role of molecular chaperones in misfolding diseases**

A current opinion is that the chaperones play important roles in the protein misfolding diseases since they are parts of the control system in the cell [76, 77]. All proteins associated with the classical amyloid diseases are secreted proteins and will therefore pass the quality control checks within the ER, where they interact with a number of proteins facilitating protein folding. In some cases, misfolded proteins are accumulated in the ER [5]. This accumulation causes “ER-stress”, a condition that normal cells respond to by increasing the transcription of genes encoding ER-chaperones, such as BiP, to facilitate protein folding or by suppressing the mRNA translation to synthesize proteins. These mechanisms are called “the unfolded protein response” (UPR). Once proteins are aggregated into extracellular amyloid deposits they are quite resistant to degradation.
The ER, cellular stress and cell death

The ER is a membrane bound cellular organelle, consisting of tubules, vesicles and cisternae. The environment is oxidizing, which facilitates formation of disulphide bonds in maturing proteins and thereby stabilizing their structures. ER is involved in protein translation, folding, post translational modifications and quality control of proteins that are to be secreted from the cell. The majority of secreted or plasma membrane proteins enter the ER and fold within it. The vesicles of the ER are responsible for transport of proteins to be used in the cell membrane or to be secreted from the cell. Molecular chaperones and folding enzymes assist nascent proteins to fold inside the ER and correctly folded proteins are transported to the Golgi apparatus. Proteins that are not able to fold or that are misfolded, are accumulated in the ER since they cannot be exported. There are different mechanisms responding to accumulation of unfolded or misfolded proteins inside the ER. One of the mechanisms is termed ER-associated degradation (ERAD), which recognizes the misfolded proteins and retrotranslocates them to the cytoplasm and send them for degradation by the ubiquitin-proteasome degradation machinery [78]. Another mechanism that responds to accumulation of unfolded proteins in the ER is the unfolded protein response (UPR). Accumulation of unfolded proteins in the ER may also lead to cell death (apoptosis), if the condition is prolonged and cannot be solved (Figure 10). ER chaperones and ER components play a crucial role for recognition of unfolded proteins and are continuously expressed in the ER. [79].
Figure 10) The ER functions. Proteins entering the ER are facing different destinies. The correctly folded proteins are sent for export, whereas proteins that are not able to fold are sent for degradation. Accumulation of incorrectly folded proteins leads to ER stress, which in turn can result in apoptosis if the condition is prolonged. Most ER processes involve several chaperone systems as indicated in the figure.

The unfolded protein response (UPR)

ER has a certain loading capacity, which varies between different cell types and during a cell’s life. When unfolded proteins are accumulated in the ER, the cell becomes stressed and the folding machinery gets perturbed. Unfolded proteins have hydrophobic residues exposed, which normally are buried in the interior of the folded protein. These hydrophobic parts tend to form (protein) aggregates that are toxic to cells. ER stress can also occur as a result of starvation, virus infections or heat, and other outer factors that influence cells negatively, and the condition is either transient or permanent. The cells respond to the stress by activating a pathway of signals leading to transcription of more chaperones, e.g BiP. Simultaneously, the translation of new proteins and the loading of proteins into ER are reduced, and further accumulation of unfolded proteins is decreased. The phenomenon is called the
unfolded protein response (UPR) [80, 81]. UPR is a cytoprotective phenomenon, but if the condition is prolonged, it can lead to activation of caspases and ultimately apoptosis.

ER stress leads mainly to three sets of responses: first, the amount of unfolded proteins that enters the ER is reduced (lowered protein synthesis and translocation into the ER); second, the ER folding capacity is increased (transcriptional activation of UPR target genes) and third, if the homeostasis has not been re-established, cell death (the cells commit suicide (apoptosis) to protect the organism). ER stress leads to activation of different signaling pathways, mediated by trans-membrane proteins, so called stress transducers, which sense the ER overload and transmit a signal to the cytosol where the transcription and translation of proteins take place. Three pathways have been identified (Figure 11), mediated by inositol-requiring protein-1 (IRE1), activating transcription factor-6 (ATF6) or protein kinase RNA (PKR)-like ER kinase (PERK) [82].

Ire1 was the first stress transducer to be identified. It is a trans-membrane protein with an ER luminal domain, which can bind to BiP or unfolded ER proteins, and a cytoplasmic domain which has a site for protein kinase activity. Ire1 is called Ire1p in yeast and Ire1α or Ire1β in mammals [83]. When unfolded proteins are accumulated inside the ER, BiP is released from the luminal domain of Ire1, leading to oligomerization and trans-autophosphorylation of Ire1. The trans-autophosphorylation leads to activation of Ire1’s effector function which causes double cleavage of mRNA that encodes a transcription factor called Hac1 (in yeast) or XBP1 (in mammals) leading to excision of a small fragment. The remaining parts of the mRNA are ligated and can thereafter work as an activator of UPR targets [82].

The ATF6 pathway involves ATF6α, which is a membrane spanning protein with a stress sensing domain in the ER lumen. The stress sensing domain binds to BiP and when unfolded proteins are accumulated in the ER, BiP is released, which makes it possible for ATF6α to travel to the Golgi. In the Golgi, ATF6α is cleaved, which results in a released fragment called ATF6f. After its release, ATF6f moves from Golgi to the nucleus, binds to DNA and activates gene expression. Both the Ire1/XBP1 and ATF6α pathways lead to increased folding capacity in the ER [82-84].
The third mammalian UPR pathway is via PERK, which is an ER localized transmembrane protein with homology to Ire1 [85]. Activation of PERK is through dissociation from BiP, which leads to dimerization and trans-autophosphorylation (similar to Ire1). PERK has also a cytosolic domain with kinase activity. Activation of PERK leads to phosphorylation of eIF2α, which in turn inhibits protein synthesis. Translation of a trans activator of UPR, the activating transcription factor 4 (ATF4), is on the contrary induced when eIF2α is phosphorylated [83].

Figure 11) The unfolded protein response with a central role for BiP.
**Apoptosis**

Sometimes, cells have to die. They can do it in different ways and for different reasons. One reason for cell death is tissue damage, which results in a process called necrosis. During necrosis, damaged cells swell and burst and release their contents to the surrounding area, which in turn can damage the neighbouring cells and give rise to an inflammation.

Apoptosis means programmed cell death and was first described in 1972 by Kerr and colleagues [86]. The phenomenon is distinguished from necrosis since it a genetically controlled process, mediated by proteins called proteases. Proteases have the ability to cut other proteins into pieces. And the proteases involved in apoptosis are called caspases. The role of apoptosis is to eliminate unhealthy or unnecessary cells from an organism without release of harmful substances to the surrounding cells. In apoptosis, the cells shrink and form something called apoptotic bodies. These in turn can be digested by other cells. Apoptosis is necessary for an organism to be healthy, and to regulate the balance between cell death and cell renewal in mammals by destroying excess or damaged cells. Failure in the apoptotic cascade may therefore have fatal consequences. For example, in 50-75% of all cancers, the apoptotic signal that normally regulates division of genetically damaged cells is lost, and cancer cells can continue to accumulate. Apoptosis can be blocked with HSPs, since they have been shown to interfere with caspases [61]. Depletion of Hsp70 can trigger apoptosis through the caspase 3 pathway, without other stressful stimulus [87].

**Caspases**

Caspases is a family of calcium dependent cysteine proteases and they are able to cleave their substrates after aspartate residues. Robert Horwitz and colleagues identified a gene (in C.elegans) called Ced-3, which coded for a protein with similar properties to the, by then, only known caspase (caspase 1) and what they found was required for cell death [88]. After that discovery, other caspases in different organisms were soon identified and their roles were surveyed [89]. Caspases contain three domains; an N-terminal domain (which vary in size between different caspases), a large domain containing the active site and a small C-terminal domain. Between the different domains, Asp cleavage sites are situated. When caspases are cleaved at the
Asp residues, they also become activated. Once a caspase has been activated, it starts to cleave its substrates at their Asp residues. Since they both are activated when they are cleaved at their Asp residues and activate their substrates by cleaving at Asp residues, they can activate each other. An active caspase is a tetramer composed of two large domains and two small domains, and has two catalytic sites (Figure 12). Cleavage of the substrates have effects on the cell morphology, which is the hallmark of apoptosis [90].

Caspase 3 (also known as CPP32, apopain, or YAMA) has been identified as a key mediator of apoptosis in mammalian cells. It is the most apoptotic protein and is synthesized as an inactive proenzyme. Caspase 3 is activated by cleavage at the Asp28/Ser29 bond and the Asp175/Ser176 bond. The activated caspase 3 cleaves and activates other caspases (e.g. caspases 6, 7 and 9), and there are also different targets in the cells that act as substrates for caspase 3 (e.g. poly–adenosine diphosphate ribose polymerase (PARP) and gelsolin).

Figure 12) The inactive procaspase and the active caspase.
**Methods**

**Cloning, mutagenesis**

To make the TTR mutants used in this thesis, and to generate cloned fragments of the TTR gene for cloning into specific plasmids required for transgenic cells, we performed site directed mutagenesis, using the polymerase chain reaction (PCR) technique. The PCR technique uses a cycling program, alternately heating and cooling the PCR sample in a defined series of temperature steps. At a specific temperature, the double stranded DNA helix separates and permits DNA synthesis with help from the enzyme pfu turbo DNA polymerase. To perform the site directed mutagenesis, we used the Quikchange site directed mutagenesis kit from Stratagene.

**SDS-PAGE and Western blotting**

In this work, Sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) was used to detect interactions between BiP and different TTR variants and to determine the stoichiometric composition of BiP-TTR complexes. Western blotting (based on antibody recognition of specific proteins) was used after immunoprecipitation experiments to investigate whether BiP was pulled down with TTR D18G and vice versa.

**Circular Dichroism**

All optical spectrophotometric methods are built on the principle that electromagnetic radiation *i.e.* light; interacts with electrons in a molecule. A molecule that is radiated will absorb the light if the energy in the radiation is high enough to excite an electron from its ground state to an excited state. Circular dichroism (CD) means that a
substance differently absorbs left and right vectors of circularly polarized light to different amounts. The substance that have the ability to do so are chiral chromophores e.g. the peptide bond within a protein.

Circular Dichroism of proteins can be measured at wavelengths where light absorption takes place. When the dichroism is measured as a function of wavelength, a CD spectrum is obtained. CD spectra can be divided into far UV (≤ 240 nm) or near UV (~ 240 – 300 nm). Near UV CD is used to study tertiary interactions within a folded protein and provides a fingerprint for the specific protein. In the near UV region, aromatic side chains (Trp, Tyr, Phe are the reporting chromophores). Far UV is used to study the secondary structure within a protein.

Fluorescence spectroscopy

Fluorescence occurs when a molecule absorbs light of a given wavelength and thereafter emits light of another, longer wavelength. The process occurs in certain molecules, called fluorophores, which often are based on a polyaromatic hydrocarbon scaffold. When a photon is absorbed by the fluorophore, an electron is excited from its lowest energy level (its ground state), to a higher energy level (excited state), and stays there for a finite time. When the electron returns to its ground state, energy is released as an emitted photon. The emitted photon has lower energy than the absorbed photon because energy is lost during the excited state lifetime, and is therefore of longer wavelength. Under optimal conditions the fluorescence signal is proportional to the concentration of the excited molecule. Fluorophores can either be intrinsic (naturally present in the molecule; Trp residues are the major intrinsic fluorophores in proteins) or extrinsic (non natural molecules that can be bound at specific sites in proteins). In this work, the fluorescence technique has been used to characterize different conformational states of TTR under the oligomerization process. In the first paper, four different molecular probes were used; 1 anilinonaphthalene-8-sulphonate (ANS), 4,4-bis-1-phenylamino-8-naphtalene sulphonate (bis-ANS), 4-(dicyanovinyl)-julolidine (DCVJ) and thioflavin T (ThT). In the second paper we used site directed insertion of pyrene. Fluorescence measurements were performed with a Hitatchi F-4500 spectrofluorometer equipped with a Xe-lamp and thermostatic cell holder. Time resolved fluorescence was performed using a an IBH (Glasgow, UK) 5000 U
fluorescence lifetime spectrometer system with 1 nm resolved excitation and emission monochromators (5000 M), equipped with a TBX-04D picosecond photon detection module with IBH laser.

**Chemical cross-linking**

Chemical cross-linking with glutaraldehyde is a method that can be used to determine the oligomerization state of a protein under different conditions. Glutaraldehyde is frequently used for induction of covalent cross links between proteins because of its ability to react with free amino groups, predominantly lysines. The results from the cross-linking reactions were analyzed by SDS-PAGE. In paper I and II, chemical cross-linking was performed to assay the rate of the oligomerization process.

**Transmission Electron Microscopy (TEM)**

The principle for TEM is the same as for an ordinary light microscope. However, in TEM, there are electrons instead of photons, the light source is an electron gun instead of a light bulb and there are magnetic lenses instead of glass lenses. The first electron microscope was invented and designed in 1933 by Ernst Ruska, who later was awarded the Nobel Prize in Physics for his finding (in 1986). TEM can be used to study different properties and structures of both physical and biological materials. A resolution of 2 Å can be achieved under optimal conditions. The electron gun creates a high intensity electron beam focused on the sample (condensor lenses collect the beam and keeps it focused when it hits the sample). To obtain an image, the electron beam must interact with the sample and provide contrast. Generally, the difference in mass number provides the contrast since a material transmits a different number of electrons depending on how electron dense it is (i.e. the heavier a material is, the fewer electrons are transmitted). Thus, the TEM image is a gray scale pattern where the darker areas correspond to those areas in the sample that scatter electron more than the lighter areas. Proteins mostly consist of carbon and are therefore stained with the much heavier uranyl acetate or phosphotungsten acetic acid during sample preparation, since the samples are analyzed on carbon coated copper grids. In paper I and II, TEM was used to study the morphology of different oligomerization states of TTR.
**Affinity chromatography and immunoprecipitation**

Affinity chromatography is a method used to purify and isolate a protein from a mixed sample. This is performed by loading a column with a matrix containing a ligand that the protein of interest will interact with and bind to. Non binding proteins can thereafter be washed out and the protein of interest can be eluted and collected. In this work, we used an Ni-NTA-affinity chromatography column which has the ability to bind to imidazol and hexa histidine tags (6-His), which is described in paper III. The column was used to purify or isolate 6-His-BiP and ligands bound to it. Immunoprecipitation was performed using anti-TTR and anti-Flag-BiP antibodies immobilized on sepharose beads, to pull down either TTR with Flag-BiP or BiP with TTR.

**Analytical ultracentrifugation**

Analytical ultracentrifugation was used to determine sizes of protein complexes, and this technique was used in paper III. The principle of analytical ultracentrifugation is that molecules sedimentate at different rotation speeds depending on their size due to the applied gravimetric field. Equilibrium sedimentation experiments were run at different rotation speeds until equilibrium was reached at each rotation speed, while monitoring absorbance at 280 nm as a function of sample depth. All sedimentation equilibrium data were obtained on a temperature-controlled Beckman XL-A/ XL-I analytical ultracentrifuge (Beckman Coulter, Fullerton, CA) equipped with an An50Ti rotor and a photoelectric scanner. Evaluation of the data was performed using the Beckman XL-A/XL-1 data analysis software applying the self-association model.
Results

The findings will be presented in four papers, which are summarized below. The first paper is a study about TTR and its misfolding and fibrillation pathway. The oligomeric intermediates in the process were studied and characterized. In paper II, the different states in the TTR oligomerization pathway were captured, and added to neuroblastoma cells to elucidate which species were toxic to cells when applied from the outside. We could see that early oligomers were toxic to neuroblastoma cells. This resulted in apoptosis and release of BiP into the cytoplasm. In the third paper, the misfolding of TTR is studied from a disease point of view and the role of BiP in misfolding diseases is discussed. The most unstable TTR variant found to date, TTR D18G, was used as a model for the study and we found that BiP strongly interacted with this mutant, which was not the case for TTR wt or other mutants. Paper IV is a study of the role of BiP for TTR D18G misfolding within a eukaryotic cell. Most of the work done previously was in vitro studies and measurements were performed on purified proteins, made in E.coli cells. However, the fourth study was done in an in vivo context, to get a cell biological aspect of the work. Human kidney cells were used to express proteins and the interactions inside the cells were studied. We could see that the in vivo results in human cells correlated well to what we had seen earlier from E.coli expressed complexes.
**Paper I**

The purpose with the study in the first paper was to characterize and understand the aggregation process of TTR. We used different techniques to detect structural changes in the aggregation process. An *in vitro* protocol for creating TTR oligomers was used. Oligomers were studied by using fluorescence spectroscopy, circular dichroism, chemical cross-linking and transmission electron microscopy.

The process for making amyloidogenic oligomers was initiated by unfolding the native, tetrameric TTR in 10 mM HCl, pH 2. The aggregation pathway was thereafter induced by addition of NaCl, giving a final NaCl concentration of 100 mM. Salt induced aggregation is a simple way for creation of soluble aggregates where addition of a high concentration of salt to acid unfolded monomeric TTR leads to immediate shielding of the positive charges in the protein which in turn change the structure of the protein. The modified structure is called the A-state [91] and is molten globule like and has a high β-sheet content (higher than in native TTR). As soon as the A-state is formed, the aggregation process takes place. This is a much cleaner experiment to detect oligomers than starting with the tetrameric protein exposed to pH 4.4 [91]. Therein the rate determining step; tetramer dissociation, and it also produces precipitated protein which is difficult to study by spectroscopy.

**Cross-linking to probe formation of aggregates**

Chemical cross-linking using glutaraldehyde was used to study formation of aggregates over time. After 1-2 hours, soluble aggregates of molecular weights around 300- 500 kDa were detected. After 2 hours, very large aggregates had formed with molecular weights > 1000 kDa (Figure 13).
Figure 13) Analysis of cross-linked TTR aggregates. A) The SDS PAGE gel demonstrated that already after 5 minutes, the aggregation process had gone far and large oligomers had been formed. B) Quantification of the bands showed that more than 50% of the protein was aggregated after 5 minutes and after 15 minutes, more or less all TTR molecules were in aggregated forms.

Size and morphology of aggregates and protofibrils

Transmission electron microscopy (TEM) was used to investigate samples taken from a TTR aggregation reaction. Samples were collected after 30 minutes, 2 hours, 24 hours and 430 hours. Small aggregates were visible after 2 hours (<10 nm length and <5 nm wide), after 24 hours small fibrillar structures had developed with sizes around 20-80 nm long and 3-8 nm wide, but there were also spherical clusters of protein aggregates with diameters of 30-50 nm present in the sample. After 430 hours, the fibrillar structures had increased in length to 100 – 200 nm and the spherical clusters had shrunk to 20-30 nm in diameter (Figure 14A).
Figure 14) From oligomers to fibrils. A) TEM micrographs demonstrated that in the beginning of the TTR aggregation process, small spherical structures had formed that later in the process shrunk and fibrils had grown in length, resulting in long fibril like structures. B) Schematic illustration of the process.

Characterization of TTR conformers using molecular probes

To kinetically detect different states of the TTR aggregation process, we used four different molecular probes (Figure 15): 8-anilino-1-naphthalene sulfonic acid (ANS), 4-4-bis-1-phenylamino-8-naphthalene sulfonate (Bis-ANS), 4-(dicyanovinyl)-julolidine (DCVJ) and thioflavin T (ThT). The four probes all have ability to bind to misfolded TTR and are useful tools for following the aggregation process of the protein.

8-anilino-1-naphthalene sulfonic acid (ANS) is a hydrophobic, charged and fluorescent molecule. Hydrophobic interaction of ANS with proteins is a widely used
method for characterizing partially folded states of proteins. The A-state of TTR binds to ANS [91]. In the thyroxin binding cavities in native TTR, ANS can also bind (and also other small molecules). Labeling of TTR with ANS or bis-ANS showed that bis-ANS did not completely fit into the thyroxin cavity but worked better than ANS when reacted with an aggregated A-state TTR. DCVJ is a molecular probe belonging to a class called molecular rotors with a unique sensitivity to torsional rigidity of the surrounding medium. We have used it as a TTR amyloid detector. ThT is the most widely used fluorescent detector of amyloids, whose action still not is completely understood [92].

Figure 15) Four different molecular probes were used to characterize different TTR conformers.

**Different kinetics for different probes**

The fluorescence response when different probes bound to unfolded monomeric TTR, native tetrameric TTR and different stages in the oligomerization process varied depending on the probe. Three probes; ANS, DCVJ, and ThT, showed increased fluorescence as the aggregation process proceeded, Bis-ANS on the contrary showed less fluorescence throughout the aggregation process (Figure 16). ANS and Bis-ANS also bound to native, tetrameric TTR. Both ThT and DCVJ could detect both early oligomers and mature fibrils with the strongest signals from the mature fibrils.
Figure 16) Different probes were used to follow the TTR misfolding reaction. Aliquots of the aggregation reaction of TTR were withdrawn and assayed at 2μM probe + 2 μM TTR. Symbols: ANS (circles), Bis-ANS (inverted triangles), DCVJ (triangles) and ThT (squares). The fluorescence intensity of the different probes in the presence of the unfolded monomer state and the burst amplitude from the fit is indicated with horizontal lines labeled with the letter U and “burst” in colors corresponding to the probe.

**Paper II**

In paper I, we characterized different states in the TTR aggregation pathway. In paper II, we harvested the different states in the aggregation pathway (during fibrillation) and challenged with neuroblastoma cells with these species. We exposed the cells to both early, oligomeric TTR species, long and mature fibrils of TTR and native TTR wt. We also wanted to investigate if BiP was upregulated in cells as a marker for UPR activation when exposed to TTR oligomers and used immunostaining for BiP to detect BiP localization in cells.

**Early oligomeric species of TTR kill human cells**

Phase contrast images of human SH-SY5Y neuroblastoma cells exposed to early oligomers or mature fibrils of TTR in 48 hours, revealed that cells that had been exposed to oligomers were dying, whereas cells exposed to mature fibrils were still alive and healthy (Figure 17). Results from MTT and caspase 3 analysis correlated well with the cell morphology and we concluded that the toxicity pathway was apoptotic. Surprisingly, TTR purified and stored at 4°C was also highly toxic, whereas
Figure 17) Phase contrast pictures of cell viability after exposure to TTR. Cells exposed to early TTR oligomers, which had aggregated for 5 min, demonstrated apoptotic morphology, such as decreased cell size and more sparse population compared to non exposed cells (C) or cells exposed to native TTR wt, 22°C (wt). Cells exposed to TTR that had aggregated for longer than 1 h were more dense. Cells exposed to TTR that had aggregated for more than one day (24 h and 1 week (1 w)) showed similar morphology as control cells (C).

TTR purified at ambient temperature (22°C) was not. The TTR tetramer is labile under cold temperatures and our results indicate a possible role for an alternate disordered tetramer toxicity or that dissociation of the tetramer into monomers render this toxicity.
Figure 18) BiP is upregulated in cells stressed with TTR oligomers and cold, native TTR wt. A) Confocal microscopy images of cells immunostained for BiP (green). Top micrograph: cells exposed to vehicle (C). Middle micrograph: cells exposed to early TTR oligomers (5 min). Bottom micrograph: cells exposed to cold native TTR wt (wt 4 °C). B) Western blot analysis of BiP expression in cells following exposure to early oligomers of TTR or cold native TTR wt (4 °C). GAPDH was used as a protein loading control to quantify the level of BiP expression.

Early oligomeric species of TTR induce ER stress

BiP was used as a marker for ER stress. Elevated expression of BiP and cytoplasmic localization of BiP were detected in cells challenged to early TTR oligomers or cold native TTR wt. Immunostaining of BiP showed a diffuse staining of BiP in untreated cells. In cells exposed to early TTR oligomers, BiP was more granulated and brighter stained than untreated cells and displayed a cytoplasmic staining pattern (Figure 18A). These results strengthen the hypothesis that BiP has a cytoprotective role in misfolding disease. Cells treated with cold tetrameric TTR also displayed increased immunoreactivity towards BiP but showed less significant cytoplasmic staining than the oligomer treated cells (Figure 18A). The Western blot verified an increased
expression of BiP after exposure to TTR oligomers (Figure 18B) and for cells exposed to cold tetrameric TTR compared to control cells.

**Paper III**

To understand how the chaperone BiP could interact with an unstable, aggregation prone, protein mutant like TTR<sub>D18G</sub>, plasmids containing genes for His<sub>6</sub>-BiP or FT<sub>2</sub>-TTR<sub>D18G</sub> were introduced into *E.coli* cells grown in LB media. The proteins were thereafter expressed with IPTG, the cells were harvested and the protein containing lysate was purified on a Ni-NTA-affinity chromatography column for capturing His<sub>6</sub>-BiP. As controls, BiP was also expressed with FT<sub>2</sub>-TTR<sub>A25T</sub>, FT<sub>2</sub>-TTR<sub>L55P</sub> or FT<sub>2</sub>-TTR<sub>wt</sub> and were treated in a similar way. The protein containing cell lysate was separated on the column and fractions containing column flow through, wash buffer, and protein eluate were analyzed by SDS-PAGE.

**BiP selectively binds to destabilized variants of TTR**

It became obvious from the SDS-PAGE analysis that FT<sub>2</sub>-TTR<sub>D18G</sub> was captured by His<sub>6</sub>-BiP since strong TTR bands were observed in the elution fractions when His<sub>6</sub>-BiP was co-expressed with FT<sub>2</sub>-TTR<sub>D18G</sub> (Figure 19). On the contrary, when His<sub>6</sub>-BiP was co-expressed with FT<sub>2</sub>-TTR<sub>wt</sub> or the other TTR mutants analyzed, none or very little TTR was detected on the gel. The binding capacity of BiP to TTR seemed to be correlated to the stability of the TTR variant which in turn is correlated to previous work where relation between secretion efficiency and thermodynamic and kinetic stability was found [43].
Composition of the BiP- TTR D18G complex

The purified BiP-TTR\textsubscript{D18G} complex was further analyzed to get an idea of its molecular composition. Size exclusion chromatography using a Superdex 75 gel filtration column and analytical ultracentrifugation revealed that the BiP-TTR\textsubscript{D18G} complex was composed of a variety of species with molecular masses ranging from 336 kDa to 75.5 kDa. By calculating the ratio between the two proteins against the molecular mass of complexes, it became clear that the ratio between FT\textsubscript{2}-TTR\textsubscript{D18G} and His\textsubscript{6}-BiP increased with increasing molecular weight, showing that a mixture formed of 1:1 complexes and complexes dominated by mutant TTR over BiP.

The BiP- TTR D18G interaction

To identify which part of TTR that BiP binds, we used acid unfolded TTR wt as a template, which we cleaved with trypsin and analyzed with MALDI-TOF mass spectrometry. 97% of the TTR sequence is covered with trypsin digestion and three of the best predicted binding sites, according to the BiP score software, were separated. His\textsubscript{6}-BiP was incubated with the TTR fragments and any captured fragment was purified with Ni-NTA chromatography (binding His\textsubscript{6}-BiP). One fragment was bound
Figure 20) The binding site for BiP in the TTR molecule. The F-strand in the TTR molecule was found to be its binding site for BiP.

to BiP, identified with MALDI-TOF. The sequence of the fragment was identified using electron spray ionization (ESI) tandem mass spectrometry (MS/MS). The yielded tag was searched against NCBI database and was identified as the TTR sequence 88-103 which corresponds to the C-terminal part of the 81-103 fragment from trypsinated TTR. Interestingly, the 88-103 sequence includes the complete sequence of β-strand F in the folded, monomeric TTR (Figure 20). Since the F strand in one monomer connects to another F-strand in another monomer to form a dimer, BiP binding to F-strands would therefore compete with F-strands in monomeric TTR and compromise formation of dimers and also tetramers. The F-strand is also involved in packing of growing amyloid fibers [93]. Formation of fibrils might therefore be interrupted when BiP binds to the amyloid formation interface and kept it in a soluble form.

**BiP plays a protective role against the toxic effects of TTR D18G**

To investigate what would happen with TTR D18G bound to BiP when released from BiP, we purified the complex and then added ATP to the sample. Since BiP is an ATP dependent chaperone, it releases its substrate when treated with ATP. We followed
BiP protects from TTR D18G cytotoxicity by keeping it in a soluble form. A) Micrographs of TTR D18G complex before (upper left) and after (upper right) addition of ATP. ATP releases substrates from BiP, and release of TTR D18G results in aggregation. B) ThT fluorescence of BiP/TTR D18G (filled bars) and BiP (open bars) before and after addition of ATP or addition of the competitive peptide 88-103 TTR. Without incubation (w/o inc), without ATP, 37°C incubation for 18 h (37°C no add), with ATP, 37°C, 18h (37°C ATP), with 88-103 TTR peptide, 37°C, 18h (37°C 88-103).

the process with transmission microscopy, ThT binding and SDS-PAGE before and after treatment with ATP. Release of TTR D18G from BiP resulted in formation of visible, insoluble aggregates that not could be seen when it was in complex with BiP. We also treated the BiP/TTR D18G complex with the peptide TTR 88-103 to see if it could compete with TTR D18G for BiP binding sites. The competing peptide resulted in a small amount of released TTR D18G which was detected with ThT fluorescence. We received about 30% increase of ThT fluorescence compared to the sample that was not incubated with peptide (Figure 21B). We assume that BiP plays a protective role against the toxic effects of TTR D18G by maintaining TTR in a soluble, here benign, oligomeric form.
Paper IV

In this paper, the idea was to obtain a cell biological aspect of BiP/TTR D18G binding and to investigate if the *E.coli* derived complexes could be confirmed in a human cellular system. We wanted to study complex formation between BiP and TTR D18G *in vivo* and used human 293T kidney cells to overexpress the proteins. We also wanted to investigate if BiP would influence the degradation rate of TTR D18G.

We could clearly see that the complex was formed *in vivo* in human cells, which confirmed our previous results. It is also known from before that BiP has the ability to oligomerize. We found that BiP did not prevent aggregation of TTR D18G, but rather oligomerized with it, both in soluble and insoluble aggregates. Surprisingly, aggregates seemed to accumulate inside the ER. We also found that the degradation of TTR D18G was altered in presence of a high amount of BiP.

BiP interacts with TTR D18G in the mammalian ER

In paper IV, we transfected human kidney cells with TTR D18G or BiP and thereafter analyzed the cell lysate using immunoprecipitation and western blotting (Figure 22). Both soluble and insoluble proteins were analyzed and both BiP and TTR D18G were present in both fractions. BiP seemed to increase the amount of soluble TTR D18G when they were co-expressed. BiP appeared to co-fractionate with aggregated TTR D18G, this is also in accordance of what we found in paper III.

The degradation rate of TTR D18G is slowed down in the presence of BiP

Since TTR D18G is extremely aggregation prone and appeared to accumulate in the ER, we were interested in investigating its degradation rate in live cells. Pulse chase (S$^{35}$) analysis of transfected 293T cells with TTR D18G or TTR D18G and BiP were performed and showed that overexpression of BiP reduced the degradation rate (Figure 23).
Figure 22) Selective binding of mutant TTR. Cells expressing Flag-BiP and/or TTRwt or TTR D18G were lysed with triton. The triton supernatants (containing the soluble protein fractions) and the triton pellets (containing the insoluble protein fractions) were collected. Immunoprecipitation with anti-TTR antibody shows that Flag-BiP is pulled down with TTR D18G and to a small extent with TTR wt. The amount of soluble TTR D18G seemed to increase when BiP was overexpressed.

Figure 23) The TTR D18G degradation rate is slowed down in presence of BiP. Pulse chase analysis results showed that the degradation of TTR D18G alone (circles) occurred faster than when BiP was overexpressed in the cells (squares).
The BiP- TTR D18G complex was present in a wide distribution of molecular weights

In paper III, we showed that soluble complexes of BiP and TTR D18G, purified from *E.coli* bacteria, existed as a broad distribution of molecular weights. In paper IV, we analyzed both the soluble protein fractions, and the insoluble protein fractions from eukaryotic cells overexpressing TTR D18G and BiP. A glycerol gradient was performed to determine the size distribution of oligomers in the soluble fractions, and the influence of BiP on oligomer size (the insoluble fractions were treated with SDS and DTT in order to denature them; the results were investigated using SDS-PAGE and Western blotting with TTR antibody). We found that both BiP and TTR D18G were abundant the soluble fractions and that for the TTR D18G fractions expressed alone, the large oligomers dominated and also a significant amount of small oligomers were present. For the TTR D18G protein co-expressed with BiP, the distribution of molecular weights was evenly spread throughout the glycerol gradient. Hence, BiP appear to shift large oligomers towards intermediate size oligomers.

Figure 24) Distribution of oligomers in cells expressing TTR D18G alone or TTR D18G and BiP.
**BiP can protect cells from TTR D18G cytotoxicity**

In paper II, we worked with neuroblastoma cells to investigate the cells viability after exposure to oligomeric species of TTR. We found that early oligomers were toxic to the cells, which died in an apoptotic like manner. In paper IV, we wanted to study the influence of BiP on toxic oligomeric TTR species and used the used the same principles as in paper II but exposed the cells to TTR D18G instead of TTR wt oligomers and mixed cold TTR D18G with cell medium and challenged NBC cells. To compare with, we used the cold BiP:TTR D18G complex and cold TTR wt. Phase contrast pictures of human NBC showed that cells exposed to TTR D18G were dead after 48 hours, whereas cells exposed to TTR D18G in complex with BiP were more viable. Analysis of DNA fragmentation inside the cells nuclei which is a hallmark of apoptosis revealed that TTR D18G induced apoptosis which was not the case for TTR D18G in complex with BiP. From this we concluded that BiP could protect the cells from TTR D18G cytotoxicity.

**Figure 25) BiP can rescue cells from dying in apoptosis.** Phase contrast pictures of human NBC exposed to cold TTR wt (wt), TTR D18G (D18G) or TTR D18G in complex with BiP (D18G + BiP) showed that cells treated with TTR D18G were dead after 48 hours (decreased cell size, modified morphology and sparse population) whereas TTR D18G in complex with BiP were more viable (more dense and normal morphology).
**BiP is found in TTR D18G aggregates in patient tissue**

Tissue sections from the cerebellum of a TTR D18G patient was analyzed using an amyloidotropic luminescent conjugated polymer (LCP) which emits light of a specific signature when bound to amyloid fibrils [94]. BiP antibody was used to detect the localization of BiP in the tissue sections and TTR antibodies were used to detect where the TTR was localized. Triple staining using LCP, anti-BiP and anti-TTR antibodies showed that BiP co-localized with the TTR containing amyloid in the brain (Figure 26).

*Figure 26* BiP co-localizes with TTR containing amyloid in the brain. Amyloid staining (LCP), TTR, and BiP are all co-localized, however there are patterns where the individual signals dominates, indicating that the amyloid composition is layered within the deposits (indicated with arrows, right panel).
Conclusions

From the studies in this thesis, it could be concluded that:

Fluorescence spectroscopy used intelligently (various molecular probes and time resolves techniques) is a very powerful tool to assay formation of amyloid and prefibrillar oligomers.

Formation of TTR oligomers during acidic conditions from the A-state occurs very fast (within minutes) after the process has been initiated. The formation of amyloid like fibrils occurs via oligomeric intermediates.

Oligomeric, intermediate species in the TTR aggregation pathway are toxic to neuroblastoma cells and cause apoptosis. Mature fibrils are less toxic. Cold stored native, tetrameric TTR is also cytotoxic suggesting an additional pathway for a labile tetramer or monomer to be toxic.

The ER chaperone BiP selectively captures the pathogenic, misfolding prone mutant of TTR; TTR D18G.

The binding site for BiP in TTR is the part in TTR that is involved in formation of the tetrameric structure and is possibly an elongation site in fibrils, comprising residues 88-103. Hence, BiP maintains TTR D18G in a soluble oligomeric form which should be a protection mechanism against oligomer toxicity.
BiP co-aggregates with TTR D18G. The larger the TTR containing aggregates are, the fewer BiP are in the complex. We ascribe this collection role for BiP as a molecular shepherd.

The degradation process of TTR D18G is slowed down in the presence of BiP.

BiP can escape the ER in complexes with TTR D18G and accumulate as extracellular amyloid in human brain.
References


