Prefibrillar oligomeric Transthyretin mutants

- amyloid conformation, toxicity and association with Serum amyloid P component

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Front cover: “Neuroblastoma cells on the run”
Ingenting är omöjligt,

vissa saker tar bara lite längre tid…
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ABSTRACT

Amyloidoses represent a heterogeneous group of diseases characterized by abnormal protein metabolism leading to extracellular deposition of fibrillar, proteinaceous amyloid in various tissues and organs of the body. To date more than 20 different proteins have been linked to diseases with amyloid depositions, of which Alzheimer’s disease and the prion-associated diseases are the most well known. Despite the origin of protein in the amyloid, the fibrils share some common biochemical and biophysical properties such as a diameter of 8-13 nm, a β-pleated sheet secondary structure packed in an ordered crystal-like way, Congo red and thioflavin binding with characteristic spectroscopic patterns and decoration of the fibrils with Serum amyloid P component and glycosaminoglycans.

The plasma protein transthyretin (TTR) is associated with familial amyloidosis with polyneuropathy (FAP) and senile systemic amyloidosis (SSA). FAP is a lethal, autosomal inherited disorder caused by point mutations in the TTR-gene. More than 80 different mutations have been associated with amyloid formation and linked to FAP. The interpretation is that amino acid replacements at different sites of the polypeptide lead to reduced stability. Mutant TTR were constructed that have a strong tendency to self-aggregate under physiological conditions. The precipitates were shown to be amyloid by staining with thioflavin T and Congo red. As the mutants were sensitive to trypsin cleavage compared to plasma TTR, we suggest that the mutants represent amyloid precursors or that they may share structural properties with intermediates on a pathway leading to amyloid deposition. Monoclonal antibodies were generated that exclusively recognize the amyloidogenic folding of TTR providing direct biochemical evidence for a structural change in amyloidogenic intermediates. Two cryptic epitopes were mapped to a domain of TTR, where most mutations associated with amyloidosis occur and is proposed to be displaced at the initial phase of amyloid formation. Amyloidogenic intermediates of TTR were shown to induce a toxic, free radical dependent, response in cultured neuroblastoma cells. Morphological studies revealed a correlation between toxicity (apoptosis) and the presence of immature amyloid suggesting that mature full-length fibrils represent an inert end stage, which might serve as a rescue mechanism.

Serum amyloid P component (SAP) is a highly conserved plasma glycoprotein universally found associated with amyloid depositions independently of protein origin. SAP’s role in amyloid formation is contradictory since both inhibition and promotion of aggregation have been shown in the case of fibril formation from the Aβ peptide of Alzheimer’s disease. Amyloidogenic prefibrils of TTR were shown to bind SAP and no interference with aggregation was detected. SAP co-localize in patches with mutant TTR on the surface of neuroblastoma cells and prevent apoptosis induced by mutant TTR and Aβ peptide, while several other molecules known to decorate amyloid fibrils were without effect.
PAPERS IN THIS THESIS

This thesis is based on the following publications and manuscript, which will be referred to by the roman numerals (paper I-IV)


Exposure of cryptic epitopes on transthyretin only in amyloid and in amyloidogenic mutants. Proc Natl Acad Sci USA. 1999 Mar 16;96(6):3108-13

III Andersson K, Olofsson A, Nielsen EH, Svehag SE, Lundgren E

IV Andersson K, Lundgren E
Inhibition of amyloid induced apoptosis – a new role for Serum amyloid P component. Manuscript
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ApoE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>APP</td>
<td>Alzheimer precursor protein</td>
</tr>
<tr>
<td>Aβ</td>
<td>Alzheimer β-peptide</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<tr>
<td>GAG</td>
<td>Glycosaminoglycans</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAB</td>
<td>Monoclonal antibody</td>
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<tr>
<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>RbP</td>
<td>Retinol binding protein</td>
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<tr>
<td>SAP</td>
<td>Serum amyloid P component</td>
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<tr>
<td>TTR</td>
<td>Transthyretin</td>
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<tr>
<td>Wt TTR</td>
<td>Wild type transthyretin</td>
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</table>
INTRODUCTION

Amyloidosis

Classical definition of amyloidosis: Amyloid diseases are secondary protein structure diseases in which insoluble protein fibrils accumulate extracellularly*. About twenty different types of fibrils have been described in human amyloidosis, each with a different clinical picture. All types of tissue amyloid consist of a major fibrillar protein (approximately 90%) that defines the type of amyloid plus a various minor components. All amyloid share certain physical and pathologic properties.

*) Lately the definition of amyloid has been extended to include intracellular fibrillar protein deposits, such as those formed by α-synuclein and tau.

Figure 1. Electron micrograph of negative stained vitreous amyloid
History

Due to early confusion concerning terminology, it is difficult to say when amyloid was first described. However, several autopsy reports from the 17th century probably describe what we today know as amyloidosis. The word amyloid is derived from the Greek word amylon and the Latin amyllum meaning starch or cellulose. The name amyloid was coined by Rudolph Virchow, a German physician, in 1854 when he found abnormal extracellular material in the liver during autopsy. Virchow described its reaction with iodine and sulphuric acid, which at the time, was markers for starch. In 1859 Friedreich and Kekule demonstrated that amyloid was protein and not cellulose. Some 70 years after Virchow’s description Congo red stain was introduced as a specific test for amyloid. Divry and associates discover that the amyloid deposits showed apple-green birefringence when stained with Congo red and viewed under polarized light. The staining procedure has subsequently been modified, but biopsy followed by Congo red staining is still the most important tool for the diagnosis of amyloidosis. With the use of electron microscopy in 1959, Cohen and Calkins showed that amyloid deposits are not as amorphous as they appear in the light microscope but are in fact built up of distinct nonbranching fibrillar subunits.

Structure

To date more than twenty human proteins are known to give rise to amyloid fibrils in vivo, see table 1. Although little sequence and structural homology (figure 2) between the amyloidogenic proteins they share some common features, including: green birefringence in polarised light after Congo red staining, rigid unbranched fibrillar structures and cross-β pattern upon X-ray diffraction analysis. The common structural properties of amyloid fibrils imply similar mechanisms of fibrilization and common features of amyloid disease pathogenesis. Mature amyloid fibrils are 5-13 nm in diameter although the molecular mass of the protein varies widely, 3-38 kDa. In the electron microscope, the fibrils seems to be assembled by lateral association of a number of smaller protofilaments, varying from protein to protein.
Table 1  Examples of amyloid diseases and the proteins involved

<table>
<thead>
<tr>
<th>Protein</th>
<th>Clinical syndrome</th>
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<tbody>
<tr>
<td>Amylin</td>
<td>Type II diabetes</td>
</tr>
<tr>
<td>Androgen receptor</td>
<td>Spinal and bulbar muscular atrophy</td>
</tr>
<tr>
<td>Apolipoprotein AI</td>
<td>Familial amyloidosis polyneuropathy III</td>
</tr>
<tr>
<td>Apolipoprotein AII</td>
<td>Hereditary renal amyloidosis</td>
</tr>
<tr>
<td>Ataxins</td>
<td>Spinocerebellar ataxias</td>
</tr>
<tr>
<td>Atrial natriuretic factor</td>
<td>Atrial amyloidosis</td>
</tr>
<tr>
<td>Aβ-peptide</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>Calcitonin</td>
<td>Medullary carcinoma of the thyroid</td>
</tr>
<tr>
<td>Cystatin C</td>
<td>Hereditary cerebral amyloidosis angiopathy</td>
</tr>
<tr>
<td>Gelsolin</td>
<td>Finnish hereditary systemic amyloidosis</td>
</tr>
<tr>
<td>Huntingtonin</td>
<td>Huntington’s disease</td>
</tr>
<tr>
<td>Immunoglobulin (light or heavy chain)</td>
<td>Primary myeloma-associated amyloidosis</td>
</tr>
<tr>
<td>Insulin</td>
<td>Injection-localised amyloidosis</td>
</tr>
<tr>
<td>Islet amyloid polypeptide</td>
<td>Type II diabetes mellitus</td>
</tr>
<tr>
<td>Lactadherin, Medin</td>
<td>Aortic medial amyloid</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>Hereditary non-neuropathic systemic amyloidosis</td>
</tr>
<tr>
<td>Prion protein</td>
<td>Creutzfeldt-Jakob disease</td>
</tr>
<tr>
<td>Serum amyloid A</td>
<td>Secondary systemic amyloidosis</td>
</tr>
<tr>
<td>Superoxide dismutase 1</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>TATA box-binding protein</td>
<td>Spinocerebellar ataxia 17</td>
</tr>
<tr>
<td>Tau</td>
<td>Fronto-temporal dementias</td>
</tr>
<tr>
<td>Transthyretin</td>
<td>Familial amyloidosis polyneuropathy, Senile systemic amyloidosis</td>
</tr>
<tr>
<td>α-fibrinogen</td>
<td>Hereditary renal amyloidosis</td>
</tr>
<tr>
<td>α-synuclein</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>β2-microglobulin</td>
<td>Haemodialysis-related amyloidosis</td>
</tr>
</tbody>
</table>
Figure 2  Different amyloid precursor proteins

The coordinates for the structures were retrieved from the Protein Data Bank. PDB ID:s 1AV1\textsuperscript{10}, 1BA4\textsuperscript{11}, 1XQ8\textsuperscript{12}, 1KCQ\textsuperscript{13}, 1LDS\textsuperscript{14}, 1HJM\textsuperscript{15} and 1CJ6\textsuperscript{16}.
**Transthyretin**

**History**

Transthyretin (TTR) was first discovered, 1942, in an electrophoresis of cerebrospinal fluid (CSF)\(^{17}\) and later also observed in serum\(^{18}\). It was first called component X and later named “prealbumin”, a name that describes its movement in front of albumin in electrophoresis. In 1958 it was demonstrated that this protein is a carrier for thyroxine\(^{19}\). Ten years later TTR was shown to bind to the retinol-binding protein\(^{20}\). In 1982, when it was very clear that TTR was not related to albumin, the name transthyretin was proposed for this protein to avoid confusion\(^{21}\). The name transthyretin describes the functions of the protein, *transport of thyroxine* and *retinol binding protein*.

**Structure**

The structure of human TTR was one of the first molecules to be solved with X-ray crystallography revealing that the native structure is a tetramer, consisting of four identical subunits\(^{22}\). Each monomer consists of 127 amino acid residues\(^{23}\), of which 57 are arranged into eight β-strands organised into two β-sheets D-A-G-H and C-B-E-F with a short α-helix between strand E and G\(^{22}\), (see fig 3, upper left). Two monomers associate with their respective F and H strands to form a dimer (fig 3, upper right). Finally, via interactions between the AB loop and the GH loop the tetramer is formed (fig 3, bottom).

**Function**

Together with thyroxine-binding globulin and serum albumin, TTR act as transport protein for the thyroid hormone thyroxine. The tetramer has a central channel which contains two binding sites for thyroxine. Due to negative cooperativity only one site is occupied at the time\(^{24}\). TTR also function as a transport protein for vitamin A by association with retinol-binding protein (RbP) carrying retinol\(^{25}\). Normally the low molecular weight of RbP would lead to renal clearance by glomerular filtration in the kidneys. Theoretically TTR contain four binding sites for RbP (one on each monomer). However, similar to thyroxine binding, one RbP molecule induce negative cooperativity\(^{26}\), even though the crystals contain one TTR tetramer and two RbP molecules\(^{27}\), see figure 4.
Figure 3. The structure of human transthyretin
Monomeric subunit (upper left), dimer (upper right) and tetramer (bottom)
The coordinates for the structures were retrieved from the Protein Data Bank.
PDB IDs: 1F41 and 2PAB
TTR in mainly synthesized in the liver\textsuperscript{29}, but low amounts is also produced by the epithelial cells in the choroids plexus in the brain\textsuperscript{30,31}, retinal pigment epithelial cells\textsuperscript{32} and by A-cells of the pancreatic islets\textsuperscript{33}. A normal plasma concentration of TTR is about 0.3mg/ml and in CSF 0.02mg/ml. The half-life of plasma TTR is 2 days\textsuperscript{34} and the main sites for degradation are liver, muscle and skin\textsuperscript{35}. TTR is not degraded within the nervous system. Plasma TTR levels is reduced in conditions with inflammation and protein malnutrition, while the level in CSF is constant.

The TTR sequence and its structure are highly conserved during evolution\textsuperscript{36}, suggesting an important role. However, TTR knockout mice are phenotypically normal, viable and fertile although they have no detectable retinol in plasma and a decreased level of thyroid hormone\textsuperscript{37}.
**Hereditary amyloidosis**

Systemic hereditary amyloidosis is a group of diseases associated with structural mutations in plasma proteins\(^3^8\). Since peripheral neuropathy is the most prominent feature of these types of amyloidosis, the term ‘familial amyloidotic polyneuropathy’ (FAP) was coined. The different forms were classified according to their clinical symptoms, before the amyloid proteins or the mutations involved were known.

There are thus four different forms of FAP, see table 2.

<table>
<thead>
<tr>
<th>Table 2  Classification of FAP</th>
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<tbody>
<tr>
<td><strong>FAP type I</strong> 39</td>
</tr>
<tr>
<td><strong>FAP type II</strong> 40</td>
</tr>
<tr>
<td><strong>FAP type III</strong> 41</td>
</tr>
<tr>
<td><strong>FAP type IV</strong> 42</td>
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</table>

**FAP type I**

Familiar amyloidosis with polyneuropathy (FAP) was first described by Andrade in 1951 as a disease occurring in the village Póvoa de Varzim, located in the northern area of Portugal\(^3^9\). The first 10 cases from Lapland and Skellefteå (Sweden) with peripheral neuropathy and vitreous opacities was reported in 1968\(^4^3\). The same year, Dr Araki reported on a family in Arao, Japan which also suffered from FAP\(^4^3,4^4\). The amyloid fibrils of the Portuguese, Japanese and Swedish patients all consisted of transthyretin\(^4^5\) and in 1985 investigators reported that methionine is substituted for valine at position 30.
(TTR V30M) in the variant transthyretin. Since then, more than 80 different mutations have been found. The majority is related to amyloid deposition but a small portion is non-amyloidogenic or even seems to have a protective effect.

When there were only a few geographical locations known where the TTR V30M mutation occurred, it was speculated that the gene might have been transmitted from Portugal to both Sweden and Japan. In all three countries the loci of the disease are in coastal villages, and there is historical evidence of contact between the countries several hundred years ago.

FAP type II
This type of FAP was described both in 1986, a large family in Indiana, (Swiss origin) and in 1989, when a pedigree comprising 11 families in Maryland, (German origin) was published. Onset is usually in the early forties with carpal tunnel syndrome as the most common initial complaint. FAP type II display a generalised neuropathy, mainly sensory in the upper limbs at first but eventually involving the lower limbs.

FAP type III
An Iowa kindred reported to have a phenotype similar to FAP type I except that a variant of ApoA1 is deposited instead of TTR V30M.

FAP type IV
This disease was first described in a Finnish kindred with corneal lattice dystrophy followed by a progressive cranial neuropathy. The fibril protein in this disease is an abnormal fragment of the plasma protein, Gelsolin.
Amyloid associated molecules

Each type of amyloid is characterised by the specific precursor protein that forms the fibrils. Despite that the amyloidogenic proteins display little sequence and structural homology, there are some components found in all amyloid deposits, irrespective of amyloid protein$^{51}$.

![Schematic drawing of amyloid and its associated molecules](modified from T. Stenstad, Sulphated Polysaccarides in Amyloidosis.)

**Figure 5. Schematic drawing of amyloid and its associated molecules**

Modified from T. Stenstad, Sulphated Polysaccarides in Amyloidosis.
Glycosaminoglycans

Glycosaminoglycans (GAG’s) are polysaccharide chains consisting of repetitive disaccharide repeats. They are categorised according to sugar composition, chain length and sulphation patterns\textsuperscript{52}. Different amyloids might exhibit variations in their associated GAG’s, depending on the type of amyloid fibril protein. Analyses of TTR amyloid deposits reveal an association with Chondroitin sulphate, Dermatan sulphate, Heparan sulphate and Hyaluronan\textsuperscript{53}.

Basement membrane compounds

Other components of the basement membrane are often found associated with amyloid deposits for instance, fibronectin, type IV collagen, and laminin\textsuperscript{54}.

Apolipoprotein E

Apolipoprotein E (ApoE) is a common constituent in amyloid deposits\textsuperscript{55} and possibly act as a pathological chaperone\textsuperscript{56}. The ApoE gene is a major risk factor for late-onset Alzheimer’s disease with the ApoE4 allele increasing and the ApoE2 allele decreasing the risk for developing AD\textsuperscript{57}.

Serum amyloid P component

Human serum amyloid P component (SAP) consists of ten identical polypeptide subunits, which are noncovalently associated in two pentameric discs, figure 6, interacting face-to-face\textsuperscript{58}. SAP is structurally related to C-reactive protein (CRP), the classic acute phase protein in humans. Both proteins are found in all mammals. Individuals unable to synthesize SAP or CRP have not yet been described, suggesting that these proteins fulfill important biological functions\textsuperscript{59-61}.

SAP is mainly produced by hepatocytes in the liver, but mRNAs is found in lung, heart, arteries, kidney and spleen. Suggesting that other cells may produce these pentraxins\textsuperscript{62}.
Figure 6. Three dimensional structure of the SAP pentameric disc
The coordinates for the structures were retrieved from the Protein Data Bank.
PDB ID:1SAC63.

SAP and CRP are members of the pentraxin family. They share 51% of amino acid identity and 59% nucleotide sequence identity. Either protein can bind C1q and activate the complement system65,66. Although in human plasma levels of SAP hardly change while circulating levels of CRP may increase up to 1000-fold during an acute phase response67.

The name pentraxin, coined by Osmand et al. in 197758, is derived from the Greek words for five (penta) berries (ragos) and reflects the arrangement of the globular subunits of SAP and CRP.
SAP does not only exist in plasma, it can also be found in amyloid deposits\textsuperscript{68–70} and in elastic fibers in blood vessel walls\textsuperscript{71}, as well as in the normal renal glomerular basement membrane\textsuperscript{72}.

SAP has the ability to bind several ligands in the presence of calcium ions, such as amyloid fibrils of any kind\textsuperscript{68}, heparin and dermatan sulphate\textsuperscript{73}, agarose\textsuperscript{74}, C4 binding protein\textsuperscript{75}, C1q\textsuperscript{76}, laminin\textsuperscript{77} and type V collagen\textsuperscript{78}. SAP also binds to phosphoethanolamine-containing compounds such as phosphatidylethanolamine\textsuperscript{79}, DNA\textsuperscript{80}, chromatin\textsuperscript{81} and histones\textsuperscript{82,83}.

It has been suggested the SAP might recognise specific features of the turns between the $\beta$-strands on the surface of amyloid fibrils. Modelling studies have shown that the double calcium ligand binding site of SAP can indeed accommodate a $\beta$-hairpin\textsuperscript{84}. That could explain why SAP binding only is seen when the amyloid proteins are aggregated into fibrils and not when they are native in solution.

The biological function in humans has yet to be elucidated. Some insight in the function of SAP was obtained in SAP-deficient mice where the majority develop antinuclear antibodies. It was interpreted as evidence that SAP is controlling the degradation of chromatin \textit{in vivo} and prevent antinuclear autoimmunity\textsuperscript{85}. Further investigations reveal that although the antinuclear antibody titer is high, the mice did not necessarily develop severe glomerulonephritis\textsuperscript{86}.

SAP is found associated to both early and late apoptotic cells\textsuperscript{87} via its ability to bind phosphatidylethanolamine. Which is exposed on the cell surface as soon as the membrane is flip-flopped. However, no further biological relevance were reported.

Lately, SAP has become an attractive therapeutic target in all amyloid-related diseases. This idea has straightened by the demonstration of prolonged half-life of SAP in amyloid deposits, 30 days compared to 24 hours in the circulation\textsuperscript{88}, the extreme proteinase resistance of SAP\textsuperscript{89} and the fact that \textit{in vivo} SAP is taken up and catabolised only by hepatocytes\textsuperscript{90}.

A drug (CPHPC) was developed that is a competitive inhibitor of SAP binding to amyloid fibrils and also crosslinks and dimerizes SAP molecules\textsuperscript{91}. In clinical studies SAP was rapid depleted from plasma when CPHPC was administrated.
In case of individuals with little or no amyloid depositions the plasma concentration of SAP returned to normal within 48 hours, but in subjects with significant amyloidosis the SAP value remained low for prolonged periods of time. This indicated that most of the newly produced SAP was distributed to the amyloid deposits before becoming available to replete the plasma pool. It is believed that depletion of the SAP bound to amyloid deposits facilitates degradation of the fibrils by proteolytic enzymes and phagocytes.

Amyloid toxicity

Until recently it was assumed that the aggregates most toxic to cells were mature amyloid fibrils, since they had been commonly detected in pathological deposits. A massive extracellular deposit of amyloid distorts tissue architecture and therefore disrupts normal functioning, ultimately leading to organ failure. However, no general connection has been established between the amount of amyloid deposited and the severity of the disease.

In the field of Alzheimer’s disease, several research groups have focused on oxidative stress in an attempt to explain amyloid toxicity. In vitro studies have consistently shown that amyloid fibrils are toxic to various types of cells and that this toxicity is coupled to oxidative stress or oxidative derived products. Although it has been reported that antioxidants have a protective role against amyloid induced toxicity, there are cases where the accumulation of intracellular reactive oxygen species or 4-hydroxynonenal is attenuated but cell death is still observed.

During the last years an increasing number of reports indicate that early aggregates are the most toxic species. The hypothesis that toxicity primarily is caused in the early stages of fibrillogenesis provides an explanation for the lack of correlation between the density of fibrillar plaques in the brain of Alzheimer patients and the severity of the clinical symptoms. Transgenic mice over-expressing APP display neurodegeneration and learning impairments before any plaque formation could be detected.
Interaction of Aβ with the cell membrane could via cell surface molecules mediate a stress response. Several possible receptors/binding sites for Aβ has been identified\textsuperscript{109-112}. At least one has a more general function. The receptor for advanced glycation endproducts (RAGE) seems to recognise β-sheet structures in several different proteins\textsuperscript{113}.

Another hypothesis is that amyloid exposes hydrophobic surfaces that favour the interaction with the plasma membrane, leading to membrane damage via the formation of non-specific ion channels or pores. These structures have been described for a number of peptides and proteins associated with amyloidosis\textsuperscript{114-117}. This theory does not necessarily reduce the importance of oxidative stress as a causative agent in amyloid toxicity. It rather suggest that channel formation could initiate disturbance in ion homeostasis that eventually leads to an amplification of a stress response.
AIMS OF THE PRESENT STUDY

I. Characterisation of amyloidogenic mutants
   – Can the constructed TTR mutants spontaneous aggregate in vitro?
   – Are there any similarities between the precipitates and amyloid?

II. Development of TTR amyloid specific antibodies
    – Do TTR amyloidogenic mutants have common epitopes?
    – Are the epitopes exposed on ex vivo amyloid?

III. Toxicity caused by TTR amyloidogenic mutants
     – Can TTR mutants induce cell toxicity?
     – Is there any correlation between morphology and cell death?

IV. Role of amyloid associated molecules
    – Can SAP associate with in vitro formed prefibrils?
    – Are amyloid associated molecules promoting or inhibiting toxicity?
RESULTS & DISCUSSION

I. Characterisation of amyloidogenic mutants

It has been suggested that point mutations destabilize the protein and facilitate self-aggregation. A model was proposed where the whole edge of the molecule, containing the C- and D- stands, loops out before TTR starts to aggregate into fibrils\textsuperscript{118}. Analysing the distribution of mutations along the TTR molecule reveals a broad peak over the same region. It was suggested to be a “hot spot” for amyloidogenic mutations\textsuperscript{119} and two mutants were constructed were the amino acids in position 53-55 (D-strand, see figure 7) were either deleted or substituted.

![Three dimensional structure of the TTR dimer](image)

**Figure 7.** Three dimensional structure of the TTR dimer

The D-strand highlighted in green.

The coordinates for the structures were retrieved from the Protein Data Bank.
PDB ID:2PAB\textsuperscript{22}.
These mutants have a strong tendency to aggregate spontaneously under physiological conditions. They migrated as broad bands larger than the wild type TTR tetramer in native PAGE and were sensitive to denaturing agents. Suggesting that the mutants were unable to form a correct tetrameric quaternary structure. Only a minor fraction of the protein solution was able to form a stable tetramer that did not aggregate further. Both Congo red\textsuperscript{120} and thioflavin T\textsuperscript{121} analyses of the precipitated mutants show typical criteria for amyloid and the mutants have previously been reported to contain cross-\(\beta\) structure upon X-ray diffraction\textsuperscript{119}. These results were confirmed by TEM of precipitate sections, however the fibrils were thinner and shorter compared to control amyloid from the vitreous. This could be explained by the fact that \textit{in vivo} the amyloid is decorated with different associated molecules.

As previously reported\textsuperscript{122}, TTR fibrils isolated from vitreous amyloid contains TTR fragments of distinct lengths: TTR 49-127 and TTR 1-48. Trypsin cleavage of amyloid from both mutants gave fragments of the same size as seen for cleaved vitreous amyloid. Mutants in ‘unaggregated’ form were highly sensitive to trypsin and resulted in complete cleavage of the protein in contrast to TTRV30M and TTRwt purified from plasma that didn’t expose the cleavage site.

We were able to show that modifications in the D-strand area of the TTR molecule resulted in structurally altered proteins that easily dissociated by detergent and displayed a trypsin cleavage site. Our results support a model for generation of amyloid where destabilising mutations that leads to loss of structural integrity in the C- and D-strands is one necessary condition\textsuperscript{118,120}. It has been suggested that proteolytic cleavage is a prerequisite for amyloid formation of TTR\textsuperscript{123} but our data indicate that cleavage rather is a consequence of it. TTR assembled into fibrils adopt a disordered structure where the structural changes lead to the appearance of a trypsin cleavage site. This model is supported by previous findings from our laboratory that only a fraction of TTR in vitreous amyloid is fragmented\textsuperscript{122}.
II. Development of TTR amyloid specific antibodies

An accepted fact in amyloidogenesis is that partial destabilisation of the tetramer facilitates assembly into fibrils\textsuperscript{124}. We have previously shown that two highly amyloidogenic mutants might represent amyloid precursors\textsuperscript{125} and reveal some of the structural changes necessary for fibril formation. The alteration in protein fold between the mutants and native TTR made us assume that cryptic epitopes, normally hidden in the native protein, are exposed. It would be possible to select for monoclonal antibodies (mAb’s) that discriminate between amyloidogenic versus native protein structure.

The substitution mutant, (TTR G53S, E54D, L55S), was used to immunize the mice. Those that developed antibodies to TTR were given a 2\textsuperscript{nd} booster injection and subsequently, hybridomas were produced according to standard procedures. After hybrid selection and cloning, the cell culture supernatants were primary screened for mAb’s with reactivity to different mutant TTR molecules. We selected five hybridomas that were further tested.

In the primary screen the proteins were adsorbed directly onto the plastic surface of the ELISA plates. This procedure causes denaturation and thereby conformational changes of the protein resulting in exposure of normally hidden epitopes. In order to screen for mAb’s with specificity for a particular fold a sandwich ELISA approach was used. A polyclonal antibody was used as a link between TTR and the plate, in this way the TTR molecules preserve their native structure.

Only two antibodies show specificity for TTR in amyloid fold and not to the native structure. TTR G53S, E54D, L55S was separated by size exclusion HPLC and fractions representing different aggregation stages were collected and separated in a native PAGE, followed by immunoblotting using the two mAb’s.

The results from the ELISA and the immunoblot indicate that the mAb’s detected cryptic epitopes not exposed on normally folded TTR, unless the proteins were partial denaturated. However, the D-strand mutants express these epitopes permanently.
By screening a TTR G53S, E54D, L55S cDNA fragment library, two distinct epitopes were identified. One mAb bind to amino acids in position 39-44 and the other to amino acids in position 56-61, figure 8.

Figure 8. Space filling model of a TTR dimer
The epitope 39-44 in green and epitope 56-61 highlighted in red.
The coordinates for the structures were retrieved from the Protein Data Bank.
PDB ID: 2PAB22.

The method we used for epitope mapping gives only a minimal estimate of an epitope126. MAb 39-44 epitope include the N-terminal part of the C-strand and the C-terminal part of the loop between B- and C- strand. This epitope is exposed on the external surface but projects towards the valley formed by the D-E loops on respective monomer. This might cause a sterical hinder towards antibody binding in the native molecule. MAb 56-61 on the other hand is buried in the native tetramer. The epitope include the N-terminal part of the loop connecting the small D-strand in the inner sheet with the E-strand on the outer loop.

Finally, we address the question whether ex vivo fibrils expose these epitopes, according to the previously proposed model where the C- and D-strand are dislocated from the molecule. Amyloid from the vitreous body of the eye can be extracted under mild conditions so the integrity of the fibrils is preserved.
and diminish the risk for a false positive result due to denaturation. Both mAb’s were shown to bind the ex vivo material, suggesting that the structural change also occur in vivo, which provide even more evidence to the partial denaturation model.\textsuperscript{118}

Further proof of a structural alteration in that area was presented by Eneqvist et. al. 2000.\textsuperscript{127} after analysing the crystal structure of the TTR G53S, E54D, L55S mutant. The ‘old’ D-strand is shifted three residues and replaced by a ‘new’ strand. The phenomenon was called ‘the β-slip’.

Both the mAb’s have also been used in an unusual ELISA set up and were able to distinguish between wt and mutated TTR in plasma leading to discovery of previously unknown TTR mutations that cause FAP.\textsuperscript{128}

### III. Toxicity caused by TTR amyloidogenic mutants

The presence of protein aggregates inside or outside cells can impair a number of cell functions that ultimately lead to cell death. In the case of AD or FAP the lesions have been correlated to lipid peroxidation of cell membranes, indicating a oxidative stress response.\textsuperscript{129} Both in vitro and in vivo data has been presenting mature amyloid as the agent that induces apoptosis. Although publications on AD transgenic mice over-expressing the Aβ-peptide points to the possibility that oligomeric prefibrils can induce toxicity.\textsuperscript{108,130}

TTR amyloid formation is known to be facilitated by destabilisation of the native protein structure, classically done by low pH. Based on the changes seen in the crystal structure of TTR G53S, E54D, L55S,\textsuperscript{127} a double mutant in the A-strand was created, TTR V14N, V16E.\textsuperscript{131} This new mutant rapidly precipitated into fibrillar like structures and appeared to be even more unstable than TTR G53S, E54D, L55S.

We addressed the question whether mature amyloid fibrils or amyloidogenic intermediates is the toxic agent. Besides TTR V30M, TTRwt and vitreous derived amyloid, the two amyloidogenic mutants, TTR G53S, E54D, L55S and
TTR V14N, V16E, were chosen for their abilities to easily form folding intermediates at physiological conditions.

A neuroblastoma cell line IMR32, were co-cultured with preaggregated proteins in different stages of the fibrillogenesis. Amyloid formation is known to be facilitated by destabilisation of the native protein structure. We generated *in vitro* fibrils in low pH, from TTRwt and TTR V30M by standard methods, but the mutants proceeded to be non toxic, likewise as the vitreous fibrils gave no response at all.

The toxic effect were monitored by TUNEL staining and compared to AFM images of the different proteins in different aggregation stages. A clear correlation could be seen between the morphology of the fibrils and the TUNEL signal. Diffuse, globular, pre-fibrillar structures caused a massive toxic reaction. This finding was confirmed by both AnnexinV staining and PARP cleavage.

Although the two mutants were able to cause apoptosis there were a huge difference in aggregation rate and ability to form fibrils. TTR G53S, E54D, L55S had a slow aggregation rate and seemed to be trapped in a stage just prior to when the toxicity is diminished. The TTR V14N, V16E mutant, on the other hand, rapidly aggregated into fibrils and caused a transient toxic effect. Electron micrographs visualise this difference in morphology. Samples were analysed after a prolonged incubation to make sure that the proteins reached their ‘end stages’ in aggregation. Both samples contain small globular structures (8-9nm) that we believe are the ones responsible for toxicity. The samples also contain straight or curved protofibrils of 200nm or more in length, predominant from the TTR V14N, V16E mutant, where the fibrils from TTR G53S, E54D, L55S were shorter.

Since the TTR V30M mutant was atoxic both as native tetramer and as precipitated fibrils (low pH), we are eager to show that this apoptotic response weren’t the consequence of *in vitro* engineered mutations. Preliminary data using the clinical relevant TTR Y114C, support our previous results.

The TTR Y114C mutant can easily be converted from a native tetramer and into prefibrillar structures just by incubating the protein solution at 55°C for a couple of days (figure 9).
Figure 9. Apoptotic response to different morphologies of TTR Y114C.
The result from this experiment is consistent with the ones obtained from our \textit{in vitro} mutants. i.e. the fibril containing pellet were harmless to the cells as well as structures smaller than 100kD. TUNEL signal was obtained only in the fraction were small globular, short prefibrils existed.

There has been reported that for some FAP cases prefibrillar deposition and cell toxicity occur before any clinical symptoms emerge\textsuperscript{403}. Others have not been able to make correlations between the amounts of fibrils deposited and the severity of the disease\textsuperscript{93}.

\textbf{IV. Role of amyloid associated molecules in toxicity}

All amyloid deposits independent of protein origin contain amyloid associated molecules besides the actually amyloid fibrils (figure 5). Serum amyloid P component and glycosaminoglycans are the most common associated molecules.

It has been known for quite a time that SAP binds to mature amyloid fibrils recognising specific motifs on the fibril surface. We previously stated that our amyloidogenic mutants, TTR G53S, E54D, L55S and TTR V14N, V16E, were prefibrils but we can show that they have the ability to bind SAP. That is strong argument that, although the material is classed as immature, they are structurally arranged in the same way as amyloid. It seems that the structural motifs involved in fibril formation are separate from the ones involved in SAP binding, since no difference in aggregation is seen in the presence or absence of SAP.

In previous experiments (unpublished) we have noticed that the TTR mutants seem to concentrate on the cell surface like patches in contrast to TTR V30M that has a more diffuse appearance (figure 10).
Figure 10. Immunostaining of TTR G53S, E54D, L55S
Panel A shows a diffuse staining of TTR V30M. Panel B and C reveal a patch like appearance of TTR G53S, E54D, L55S and TTR V14N, V16E respectively.

The cell surface localisation of TTR might be an important step in causing toxicity. There is evidence that not only $\alpha$S bind cell surface molecules, Sousa et. al showed that TTR bound to the RAGE-receptor induce activation of NF-kB$^{113}$. We raised the question if decoration of prefibrils dislocates TTR from the cell surface. To our surprise it seems that the proteins still are associated with the cells and the “patchy” stain seen with the mutants still remains but now in association with SAP.

When analysing the role of the amyloid associated molecules further, we found that only SAP had any effect on apoptosis. In a concentration twice the amount in plasma (but much lower than in amyloid deposits), SAP totally inhibited the cell damage.

Previous reports suggest that SAP get internalised and by binding DNA, histones and chromatin prevent an autoimmune response$^{85}$. That was further confirmed in SAP-deficient mice where the majority develop antinuclear antibodies$^{86}$.

Thus, there is one important fact to consider when conclusions are drawn from experiments using mice as a model: SAP act as an acute phase protein in mice but the equivalent in humans is CRP!

Our hypothesis is that SAP by binding to TTR prevents the association to a receptor or other surface molecule and thereby prevent toxicity.
FINAL REMARKS

I  Characterisation of amyloidogenic mutants
   −  Can the constructed TTR mutants spontaneous aggregate in vitro?
      +  It seems that the destabilisations caused by the mutations are enough for structural changes to occur and aggregates to form.
   −  Are there any similarities between the precipitates and amyloid?
      +  Thioflavin T and Congo red assays show typical criteria for amyloid.
      +  Ordered fibrillar structures observed in TEM.

II  Development of TTR amyloid specific antibodies
   −  Do TTR amyloidogenic mutants have common epitopes?
      +  Yes, but the areas are hidden when the molecule has its native fold.
      +  Detection of mutated TTR in plasma
   −  Are the epitopes exposed in ex vivo amyloid?
      +  Both epitopes are found on the surface of the fibrils.
III Toxicity caused by TTR amyloidogenic mutants
- Can TTR mutants induce cell toxicity?
  + Only the amyloidogenic mutants were responsible for a toxic response.
- Is there any correlation between morphology and cell death?
  + Prefibrils that were shorter and curved were detected to be the toxic agent.

IV Role of amyloid associated molecules
- Can SAP associate with \textit{in vitro} formed prefibrils?
  + Yes, SAP can bind oligomeric aggregates.
- Does SAP effect apoptosis?
  + An inhibition of cell death is seen when SAP is added to the cell cultures.
REFERENCES


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