# Protein Misfolding and Amyloid Formation

Strategies for Arevention

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Cover: Molecular modeling of $A\beta_{13\text{-}26}$ interacting with Dec-DETA (Roger Strömberg).

Protein misfolding and amyloid formation - strategies for prevention

Abstract

Most proteins need to adopt a three-dimensional structure in order to function properly. Misfolding, or inability of proteins to fold, is associated with a number of diseases. In a subset of these disorders, the misfolded protein or peptide self-assembles into stable, β-sheet rich structures known as amyloid fibrils.

Alzheimer´s disease is associated with the aggregation of the amyloid β-peptide (Aβ) into oligomers and amyloid fibrils. Aβ has a discordant, i.e β-sheet preferring, helix prone to misfold and it has been proposed that stabilization of this helix could prevent aggregation. We have designed small molecules that bind to this region and stabilize Aβ in a helical conformation. This interaction reduced fibril formation and cell toxicity of the peptide and also restored a memory-linked electrophysiological function in mouse hippocampal slices treated with Aβ. Moreover, when administered orally, these compounds had a rescuing effect in a *Drosophila melanogaster* model of Aβ aggregation.

Another protein capable of forming amyloid-like fibrils in association with disease, is the human lung surfactant protein C, SP-C, which has a discordant transmembrane helix. SP-C is expressed as a pro-protein with a C-terminal, CTC, which has a Brichos domain with unknown function. Here, we show that CTC is important for the stability and folding of the pro-protein in the endoplasmic reticulum (ER). It is able to prevent the mature SP-C from aggregating *in vitro,* and is shown to bind specifically to non-helical segments and to amino acids that have been reported to promote membrane insertion in the ER. Together these data suggest a chaperone function for CTC, targeting transmembrane regions that have not attained an α-helical conformation. CTC interacts with and reduces amyloid-like fibril formation of Aβ as well as an additional amyloidogenic peptide – medin.

In conclusion this thesis explores two new strategies for preventing protein misfolding and amyloid fibril formation. The first approach utilizes designed ligands to trap the Alzheimer´s disease associated Aβ in its native helical structure. The second employs a novel, natural chaperone that bridges folding of transmembrane regions and anti-amyloid properties.

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Dedication

To my family

Control the controllable…..

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I **Nerelius C.**★, Sandegren A.★, Sargsyan H., Raunak, Leijonmarck H., Chatterjee U., Fisahn A., Imarisio S., Lomas D.A., Crowther D.C., Strömberg R. and Johansson J. (2009). α–Helix targeting reduces amyloid β-peptide toxicity. *Proc. Natl. Acad. Sci. USA*. In press. ★These authors contributed equally to this work.

II **Nerelius C.**, Raunak, Leijonmarck H., Chatterjee U., Bazoti F., Bergquist J., Johansson J. and Strömberg R. (2009). α–Helix stabilizing ligands – a new class of Aβ aggregation inhibitors. *Manuscript*.

III **Nerelius C.**, Martin E., Peng S., Gustafsson M., Nordling K., Weaver T. and Johansson J. (2008). Mutations linked to interstitial lung disease can abrogate anti-amyloid function of prosurfactant protein C. *Biochem J*. 416 (2), 201-209.

IV Johansson H.★, **Nerelius C.**★, Nordling K. and Johansson J. (2009). Preventing amyloid formation by catching unfolded transmembrane segments. *J Mol Biol*. doi:10.1016/j.jmb.2009.04.021 ★These authors contributed equally to this work.

V **Nerelius C.**, Gustafsson M., Nordling K., Larsson A. and Johansson J. (2009). Anti-amyloid activity of the C-terminal domain of proSP-C against amyloid β-peptide and medin. *Biochemistry*. 48(17), 3778-3786.

Papers not included in the thesis:

VI   Chatterjee U., Bose, P.P, **Nerelius C.**, Govender T., Norström T., Gogoll A., Sandegren A., Göthelid E., Johansson J. and Arvidsson P.I. (2009). Poly-N-methylated amyloid β-peptide (Aβ) C-terminal fragments reduce Aβ toxicity *in vitro* and in *Drosophila melanogaster. Submitted.*

VII   **Nerelius C.**, Johansson J. and Sandegren A. (2009). Amyloid β-peptide aggregation. What does it result in and how can it be prevented? *Front Biosci.* 1(14), 1716-29.

VIII Kinghorn K.J., Crowther D.C., Sharp L.K., **Nerelius C.**, Davis R.L., Chang H.T., Green C., Gubb D.C., Johansson J. and Lomas D.A. (2006). Neuroserpin binds Aβ and is a neuroprotective component of amyloid plaques in Alzheimer disease. *J Biol Chem.* 281(39), 29268-77.

Abbreviations

Three and one letter codes for the 20 naturally occurring amino acids

Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Other abbreviations

Aβ	Amyloid β–peptide
AD	Alzheimer's disease
ADDLs	Aβ derived diffusible ligands
APP	Amyloid-β precursor protein
BACE	β-site APP cleaving enzyme
CD	Circular dichroism
CNS	Central nervous system
CTC	C-terminal part of proSP-C
DMSO	Dimethyl sulfoxide
ESI-MS	Electrospray ionization mass spectrometry
HEKcells	Human embryonic kidney cells
kDa	Kilodalton
LTP	Long-term potentiation
MALDI	Matrix-assisted laser-desorption ionization
NMR	Nuclear magnetic resonance
PC12 cells	Rat pheochromocytoma–derived cells
proSP-C	Surfactant protein C precursor
SDS	Sodium dodecyl sulfate
SEC	Size exclusion chromatography
SP-C	Surfactant protein C
TEM	Transmission electron microscopy
TFE	Trifluoroethanol
ThT	Thioflavin T

1 Introduction

1.1 Protein folding and misfolding

1.1.1 Protein folding and quality control

Proteins are among the most important molecules in living organisms, constituting more than half the dry weight of a cell. There are approximately 20,500 protein-encoding genes in the human genome (Clamp *et al.*, 2007) and these macromolecules take part in diverse processes such as food digestion and host defense.

Proteins are made up of amino acid residues linked by peptide bonds. There are 20 naturally occurring amino acids and the order in which they occur in a polypeptide chain is referred to as a proteins primary structure. The primary structure, governed by environmental conditions, directs the folding of the polypeptide and the formation of secondary structure elements such as α–helices and β–strands. These elements are further arranged in space to produce the proteins functional 3D-structure; the tertiary and quarternary structure.

Folding is a complex process still far from fully understood. There have been many attempts to predict the folding of proteins, some with great success such as the Rosetta algorithm (Das & Baker, 2008; Rohl *et al.*, 2004). However, no general solution, applicable to all proteins, has so far been achieved. Briefly, the driving force for correct folding is believed to be the search for the lowest possible energy state, a process that is governed by interactions within the molecules as well as between the molecule and the surrounding solvent. Each conformation that the protein can adopt is

associated with a free energy. Plotting free energies against their corresponding conformations generates an energy landscape on which the protein can fold on several pathways in its search for an energy minimum (Figure 1). During folding, proteins often form intermediate states, which are local minima in the energy landscape. The conformation with the lowest energy usually represents the native structure (Dobson, 2003; Dobson & Karplus, 1999).
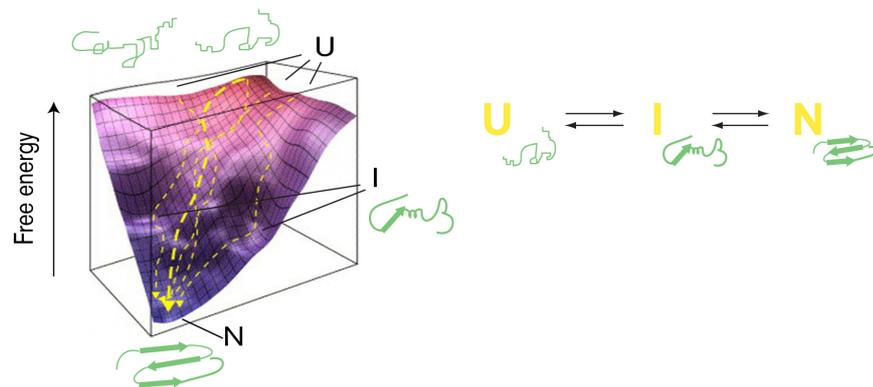

Figure 1. Energy landscape for protein folding, the unfolded (U), intermediate (I) and native states (N) are indicated. Adopted from (Tunnicliffe *et al.*, 2005) with permission from the publisher.

The paradigm stating that the function of a protein is determined by a unique 3D-structure is challenged by the existence of natively unfolded proteins. These proteins are mainly unordered but do contain some degree of secondary structure. Disordered proteins often function by binding to various partners, such as nucleic acids, and this interaction can induce folding of the unstructured protein. It is believed that the lack of stable structure could be a way of enabling the protein to have a broader binding-specificity (Fink, 2005).

The Levinthal´s paradox states that the number of possible conformations in the energy landscape for a polypeptide chain to adopt is extremely large. This would make it impossible for a protein to adopt all in a relevant time span in the search for the correct fold (Levinthal, 1968). One model put forward to explain this paradox is the hydrophobic collapse model (Dill *et al.*, 1993). In this model, protein folding is believed to start by a

hydrophobic collapse, with the burial of hydrophobic residues in a folding core. Secondary and tertiary structures are then formed within this core with interactions such as disulfide bonds and salt bridges continuing to direct the folding, further helping in the choice of conformation. This model however, may not be applicable to transmembrane proteins since they will need to expose hydrophobic parts in order to be stable in the membrane environment. Another proposed mechanism is the nucleation-condensation model (Fersht, 1997). According to this, secondary and tertiary structures form co-operatively as the whole protein condenses around a nucleus. This nucleus is formed by a few adjacent residues that have a high preference for early formation of secondary structure when stabilized by tertiary structure interactions. Together these types of constraints limit the number of available conformations and allow for the protein to fold in a biologically acceptable time span.

The crowded environment in the cell, where the concentration of macromolecules can reach 400 mg/ml (Ellis & Minton, 2003), further complicates the folding process. When the unfolded polypeptide leaves the ribosome and enters the cytoplasm, or the endoplasmic reticulum (ER), its hydrophobic domains are exposed and the crowded environment may promote hydrophobic interactions between nascent proteins and other cellular components. The failure of a protein to fold properly or to retain its fold can lead to malfunction of vital cellular processes. In some cases misfolding results in harmful aggregation. To prevent these events a number of molecular systems have evolved to assist folding, one of them being the molecular chaperones (Ellis, 1990).

Molecular chaperones provide a way of preventing aggregation and misfolding of newly synthesized proteins by maintaining them in a folding-competent conformation. They interact with the nascent chains as they are emerging from the ribosomes or take part in later stages of the folding process. Chaperones do not increase the rate of folding but rather increase the efficiency by preventing un-wanted interactions. They perform their function by recognizing and binding to structures that are normally buried in the native state e.g. hydrophobic stretches and unstructured backbone regions, thereby shielding them from the surrounding. Chaperones are found in the cytosol but also in cellular compartments like the mitochondria and the ER. The class of molecular chaperones includes many families of proteins e.g. the heat-shock proteins, like Hsp70 and Hsp 40, as well as the large complexes called chaperonins. Hsps exert their function by binding

and releasing proteins in repeated cycles during the folding event until the protein has adopted a stable structure, whereas the chaperonins have a central cavity in which folding can occur protected from other molecules (Hartl & Hayer-Hartl, 2002; Frydman, 2001; Stevens & Argon, 1999; Hartl, 1996). Recently, there have been reports suggesting that there are control mechanisms for folding in the extracellular space as well. These extracellular chaperones, including e.g. clusterin (Humphreys *et al.*, 1999) and haptoglobin (Yerbury *et al.*, 2005a), are upregulated during stress and have been shown to prevent stress-induced aggregation of a variety of proteins *in vitro* (Wilson *et al.*, 2008). The function of these chaperones is not fully known but it has been suggested that they might mediate cellular uptake and subsequent degradation (Yerbury *et al.*, 2005b).

If a protein fails to adopt a stable fold or misfolds it can form aggregates, either amorphous or in some cases structured fibrillar forms (described in the following section). Under normal circumstances this is not a major problem since the cell has efficient ways of detecting and eliminating misfolded or unfolded species before they can induce any damage to the cell. Proteins that have failed in attaining their correct fold are recognized by a cytosolic enzyme that tags them with several ubiquitin molecules. This polyubiquitin tag then targets the protein to a large multiprotein assembly, called the proteasome, where it is subsequently destroyed (Hershko & Ciechanover, 1998). Proteins that misfold in the ER are retro-translocated to the cytosol where they are then degraded by the same mechanism as described above (Meusser *et al.*, 2005; Kopito, 1997).

1.1.2 Amyloid fibrils

Despite the many mechanisms nature has generated to prevent protein misfolding and aggregation there are a large number of diseases caused by the aberrant folding of proteins, so-called misfolding diseases. In a subset of these diseases proteins undergo conformational changes and self-associate into stable, insoluble, aggregates with β-sheet structure called amyloid fibrils. These structures are highly protease resistant and the cell is often unable to degrade them. All amyloid fibrils are composed of β-sheets with their constituent hydrogen bonded strands, parallel or anti-parallel, running perpendicular to the axis of the fibril (Figure 2). This cross-β structure was first observed by X-ray diffraction (Eanes & Glenner, 1968), where amyloid fibrils show two distinct signal, one at 4.7 Å and one between 10-11 Å. The 4.7 Å signal corresponds to the hydrogen bonding distance between the

strands in each sheet and the other signal indicates the spacing between different interacting sheets. Recently, methods like solid-state NMR ((Tycko, 2006) and references therein) and cryo-electron microscopy (Serpell & Smith, 2000) have further helped in characterizing the fibril structure from a number of proteins. Mature fibrils are usually made up of 2-6 fibrillar subunits named protofilaments which, slightly twisted around each other, form unbranched fibers, 7-12 nm in diameter (Makin & Serpell, 2005). In order to be called amyloid, certain criteria has to be met. Depositions has to occur *in vivo*, the fibrils should be straight and unbranched and show the typical X-ray diffraction as mentioned above. In addition, staining with the dye Congo red should result in green birefringence in polarized light. Fibrils formed *in vitro* are referred to as amyloid-like fibrils.
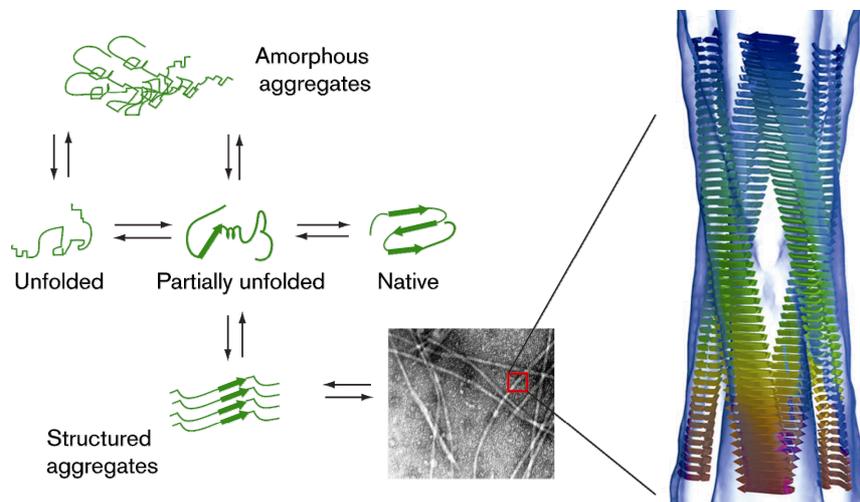
Figure 2. Protein folding and misfolding. Proteins fold and unfold continuously during their lifespan. It is believed that amorphous, unstructured aggregates can form from unfolded as well as partially unfolded states. Structured aggregates most likely form from partially structured intermediates. These aggregates can go on to form amyloid-like fibrils. These insoluble fibrils are composed of β-sheets parallel to the fibril axis and are usually made up of 2-6 protofilaments, twisted around each other. (Fibril model adapted from (Jimenez *et al.*, 1999))

There are today 28 known amyloid forming proteins and peptides in man (Benson *et al.*, 2008; Westermark *et al.*, 2007). These proteins, including e.g. the amyloid β-peptide associated with Alzheimer's disease, the islet amyloid polypeptide which aggregates in type II diabetes and the prion protein in Creutzfeld-Jakob disease, do not share any obvious sequence identities but form fibers with similar morphology. These observations

15

together with the finding that a large number of proteins not associated with amyloid disease can form amyloid-like fibrils under certain conditions, has led to the suggestion that amyloid fibril might be a generic conformation that all proteins could adopt (Stefani & Dobson, 2003). Most proteins however do not form fibrils under physiological conditions, but require harsh treatments in order to do so. Sawaya *et al.* recently proposed that the core of amyloid fibrils is a steric zipper and that the ability to form these zippers is highly sequence dependent (Sawaya *et al.*, 2007). Main determinants for fibril formation has been reported to include charge, hydrophobicity and secondary structure propensity (Chiti *et al.*, 2003). The conformational stability (Ramirez-Alvarado *et al.*, 2000; Kelly, 1996), as well as the solubility of the folded conformation, is also important for its ability to form fibrils, with fibril formation being most prominent at the pI of the protein (Schmittschmitt & Scholtz, 2003). π–stacking of aromatic residues has also been implicated to play an important role (Tartaglia *et al.*, 2004; Gazit, 2002). Amyloid diseases are classified as either localized, when only one organ is affected e.g. the brain in Creuztfeld-Jakob disease, or systemic if deposits are found in a wide range of tissues and organs e.g. lysozyme amyloidosis.

For amyloid fibril formation to occur the native protein needs to be destabilized in order for aggregation to take place. It is believed that amorphous aggregates can from both unfolded and intermediate states whereas structured aggregates like amyloid fibrils most likely originates from partially folded intermediates (Figure 2). The formation of amyloid fibrils is a nucleation-dependent process. This type of process is characterized by; a *critical concentration* below which no aggregation will occur, a *lag phase* when oligomers (nucleus) are formed from un/misfolded monomers by a series of thermodynamically unfavorable steps, followed by a more rapid *elongation phase* and finally a steady state phase where monomers and fibrils are at *equilibrium* (Figure 3). The lag phase can be abolished by adding a preformed nucleus, a so-called seed. It has been suggested that the fundamental unit of all amyloid fibrils is a dry steric zipper formed by two tightly interdigitated β-sheets (Sawaya *et al.*, 2007). The process of fibril formation then probably starts by the un-masking of several zipper-forming segments, permitting them to stack into sheets – if this is a naturally occurring event it would be compatible with the slow nucleation event.
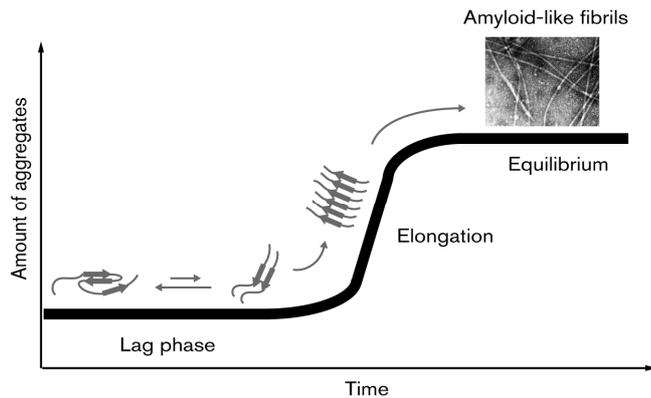
Figure 3. Formation of amyloid fibrils is a nucleation dependent process. This type of event is characterized by a slow lag-phase, followed by an elongation phase and finally a steady-state phase.

In contrast to the disease–associated amyloids, amyloid formation is sometimes utilized by nature for beneficial functions. Two examples of these functional amyloids are the human protein Pmel17 (Berson *et al.*, 2003) which forms amyloid important for skin and eye pigmentation and the amyloid fibrils called curli that are involved in cell–cell contacts of *Escherichia coli* (Chapman *et al.*, 2002).

1.2   Alzheimer´s disease

1.2.1 General introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder named after the German physician Alois Alzheimer who first described the disease in 1907 (Alzheimer, 1907). AD is the most common cause of dementia in elderly, accounting for 60-70% of all cases (Fratiglioni *et al.*, 2000). Age is the strongest risk factor for AD, the prevalence for the disease is around 1% at age 65 and around 20% at age 85 (Ferri *et al.*, 2005). Together with the growing elderly population in the world the number of people affected by AD is expected to increase enormously and with it the cost for society.

The two major neuropathological hallmarks of AD are extracellular deposits called senile plaques and intracellular neurofibrillar tangles (NFTs). NFTs are composed of paired helical filaments of hyperphosphorylated tau (Grundke-Iqbal *et al.*, 1986), a microtubule-binding protein, and are believed to form after the senile plaques (Hardy *et al.*, 1998), which are mainly composed of amyloid fibrils formed by the amyloid β-peptide (Aβ)

17

(Masters *et al.*, 1985; Glenner & Wong, 1984). Aβ is an enzymatic cleavage product generated from the amyloid-β precursor protein, and its aggregation is believed to be the major cause of the disease (Hardy & Selkoe, 2002).

The first symptoms of AD are often loss of short-term memory and as the disease progresses, disorientation, aphasia and a general decline in cognitive functions follows. Even though there are different tests and criteria available for clinical diagnosis, a definite AD diagnosis can only be made *post mortem* by a pathological exam of the brain to verify the existence of the above mentioned neuropathological lesions.

Patients suffering from the disease are usually divided into two subgroups based on age of onset. Early onset AD, which accounts for about 2 % of all AD cases, appears before the age of 65 whereas the more common form, late onset AD, called sporadic (SAD), have an onset after 65 years of age. Familial AD (FAD) belongs to the group of the early-onset AD and is caused by mutations in amyloid-β precursor protein, Presenilin 1 or Presenilin 2 (which are discussed below).

1.2.2 Amyloid-β precursor protein and the amyloid β-peptide

Amyloid-β precursor protein (APP) is a type I transmembrane protein, with its N-terminal end in the extracellular space, ubiquitously expressed in a variety of cells types (Mattson, 1997). There are three main isoforms of the protein found in the brain, APP_{695}, APP_{751} and APP_{770}, where APP_{695} is the predominant form expressed in neurons (Kitaguchi *et al.*, 1988; Ponte *et al.*, 1988; Tanzi *et al.*, 1988; Kang *et al.*, 1987). The role of APP is still unknown but a number of functions have been suggested, see (Thinakaran & Koo, 2008). Initially it was believed to be a cell surface receptor, it has been implicated to play a role in cell adhesion and also to have a trophic role, the latter function is compatible e.g. with the finding that APP is up-regulated during neuronal maturation (Hung *et al.*, 1992). A study by Kamal *et al.* suggested that the protein might be involved in axonal vesicle transport (Kamal *et al.*, 2001). A recently proposed function of APP as a ligand for the death receptor DR6 in neurons potentially links APP to Alzheimer´s disease. The study shows that an N-terminal fragment of APP is released upon trophic depravation, binds to DR6 and thereby triggers degeneration of neural cell bodies and axons (Nikolaev *et al.*, 2009).

APP is sequentially cleaved by proteases in two distinct pathways (Figure 4) (Esler & Wolfe, 2001). The so-called amyloidogenic pathway generates the amyloid β-peptide (Aβ), a peptide of predominantly 40-42 amino acid residues associated with amyloid formation and AD. In the predominant non-amyloidogenic pathway α-secretase cleaves APP within the Aβ region between residue 16 and 17 (Parvathy *et al.*, 1999; Sisodia, 1992; Esch *et al.*, 1990), releasing a large soluble fragment, αAPPs, and creating a membrane bound fragment, C83. C83 is then further cleaved by γ–secretase in the transmembrane region yielding the extracellular peptide p3 and the intracellular AICD (Haass *et al.*, 1993). γ–Secretase takes part in both the amyloidogenic and the non-amyloidogenic pathways and is a membrane associated enzyme complex made up of presenilin 1 or 2, Pen-2, Aph-1 and nicastrin (De Strooper, 2003). Mutations in the presenilins cause increase in production of $A\beta_{1-42}$ and are linked to FAD (Czech *et al.*, 2000). In the amyloidogenic pathway the first cleavage is performed by β-secretase (BACE1)(Vassar *et al.*, 1999) at the N-terminal end of the Aβ region generating C99 and the soluble N-terminal fragment βAPPs. Subsequent γ–secreatse cleavage of C99 produces Aβ. The γ–cleavage is a heterogeneous event leading to the formation of Aβ species with various lengths. The predominant species are $A\beta_{1-40}$ and the more aggregation prone $A\beta_{1-42}$ (Naslund *et al.*, 1994), but species of 38-43 residues have been observed.
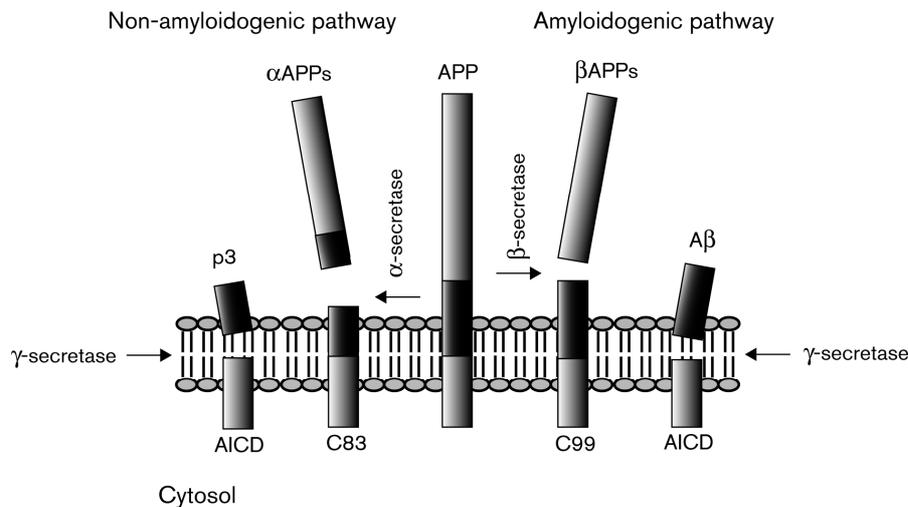

Figure 4. APP processing follows two main pathways; the non-amyloidogenic, generated by α- and γ-secretase cleavage, and the amyloidogenic, generated by β- and γ-secretase cleavage. The later pathway produces the amyloid β-peptide.

The Aβ segment corresponds to residue 596-636(8) of APP and contains a part of the transmembrane region, residues 625-638 (residues 29-42 of mature Aβ). The Aβ sequence is shown below:

$D_1AEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV_{40}IA_{42}$

The Aβ peptide has a hydrophobic C-terminal and a hydrophilic N-terminal. It is disordered in water solution but deviates from a complete random conformation by a few non-random regions (Figure 5A). NMR studies have indicated a turn-like structure at residue 8-12 and hydrophobic interactions between side-chains in the 16-24 regions with a bend-like structure at 20-24 (Riek *et al.*, 2001). The secondary structure of monomeric $Aβ_{1-40}$ and $Aβ_{1-42}$ is very similar even though their aggregation behavior is different. This difference could perhaps be explained in part by the finding that the C-terminal end of $Aβ_{1-42}$ is more rigid than that of $Aβ_{1-40}$ (Yan & Wang, 2006; Riek *et al.*, 2001) and that it shows β-strand tendency in the regions 31-36 and 39-41 (Hou *et al.*, 2004).

In membrane mimicking solutions Aβ adopts more stable secondary structures. In the presence of SDS micelles, Coles *et al.* showed the presence of two helices at positions 15-24 and 28-36 in $Aβ_{1-40}$ (Coles *et al.*, 1998). A more recent study confirmed this, indicating two helices covering the regions 15-24 and 30-35, with the first helix interacting with the micelle surface and the second one buried inside the hydrophobic interior (Jarvet *et al.*, 2007) (Figure 5B, C). The helix covering residue 16-23 is discordant, i.e. it is predicted to form a β-strand but shows a helix conformation in experimental determined structures (Kallberg *et al.*, 2001). Using structure-inducing organic solvents, more stable secondary structures are also reported but with slightly different locations. Crescenzi *et al.* showed helices at 8-25 and 28-38 connected by a β-turn in $Aβ_{1-42}$ using hexafluoro-isopropanol whereas Sticht *et al.* indicated the presence of helices in $Aβ_{1-40}$ covering regions 15-23 and 31-35 with a disordered region in-between in trifluoroethanol (Crescenzi *et al.*, 2002; Sticht *et al.*, 1995). In complex with the affibody protein $Z_{Aβ_3}$, $Aβ_{1-40}$ forms a β-hairpin, with residues 17-23 and 30-36 forming β-strands, that is similar, although the orientation of the hydrogen bonds are shifted 90°, compared to that found in mature fibrils (Hoyer *et al.*, 2008).
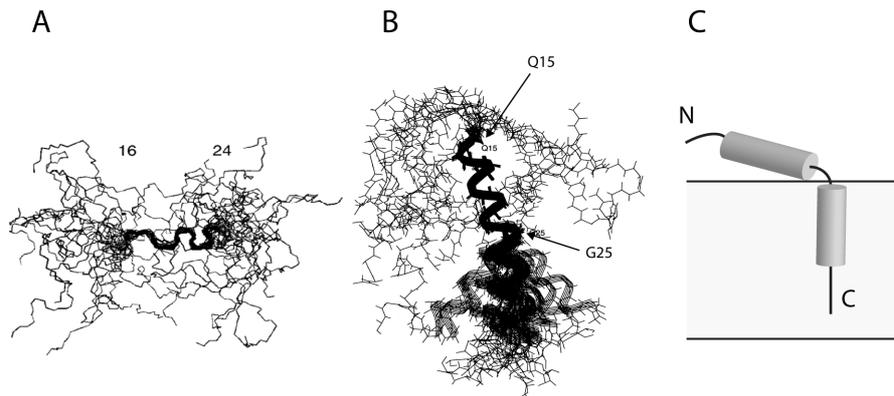
Figure 5. Models of $A\beta_{1-40}$ monomer in different solutions. A) Model structure of $A\beta_{1-40}$ in water. Twenty calculated structures, obtained by NMR constraints, are superimposed over residues 16-24 and indicate that this region contains non-random structure. From (Riek *et al.*, 2001) with permission from the publisher. B) Model of $A\beta_{1-40}$ in SDS micelles. Superimposition of residues 15-24 reveals two helices (15-24 and 28-36) with varying orientation relative to each other. From (Coles *et al.*, 1998) with permission from the publisher. C) Suggested positioning of membrane-associated $A\beta$. The barrels correspond to two helices; one in the region 15-24 positioned superficially and the other, 29-C-terminus, buried in the hydrophobic interior of the membrane. Adapted from (Coles *et al.*, 1998) and (Jarvet *et al.*, 2007).

It is believed that $A\beta$ leaves the membrane upon cleavage but it has also been suggested that it might stay and exert its toxicity from there (Marchesi, 2005). Lundin *et al.* have shown that the ability of $A\beta$ peptides to stay inserted in membranes is strongly correlated with their length and ability to adopt a helical structure (Lundin *et al.*, 2007). In their study a minimum of 46 residues was needed for membrane retention. This implies that $A\beta_{1-40}$ as well as $A\beta_{1-42}$ would be released from the membrane upon cleavage by γ-secretase. The release from the hydrophobic environment in the membrane, which probably stabilizes the helical structures, into the hydrophilic surrounding could increase the aggregation rate and fibril formation of the peptide.

1.2.3 Amyloid fibrils and oligomers

$A\beta$ forms fibrils that are deposited in the brains of affected patients in so-called plaques. The dominating $A\beta$ specie in the plaques is $A\beta_{1-42}$ (Iwatsubo *et al.*, 1994). The identification of $A\beta$ and its correlation to AD, led to the development of the amyloid cascade hypothesis in 1992 (Hardy & Higgins, 1992). In this first version, the insoluble $A\beta$ fibrillar deposits were considered to be causative of disease triggering pathogenic processes leading

to neuronal death and synaptic dysfunction. However, poor correlation between number of plaques and severity of disease has resulted in a revised version which includes soluble oligomers (Hardy & Selkoe, 2002). The level of soluble Aβ species correlates better with disease (Lue *et al.*, 1999; McLean *et al.*, 1999) and they are now believed to be the main cause of neurodegeneration.

Since fibrils were earlier believed to be the major disease-causing assemblies, much effort has been made to determine the structure of fibrils formed by $Aβ_{1-40}$ and $Aβ_{1-42}$ as well as by shorter fragments. It has been shown that fibrils formed under different conditions vary in their morphology (see e.g. (Kodali & Wetzel, 2007; Paravastu *et al.*, 2006; Goldsbury *et al.*, 2000)) but the same conditions can also give rise to a polymorphic population of fibrils (Meinhardt *et al.*, 2009). Solid state NMR, atomic force microscopy and transmission electron microscopy have been used to show that different fibril morphologies have different molecular structure indicating multiple assembly pathways (Goldsbury *et al.*, 2005; Petkova *et al.*, 2005). A possible explanation as to why fibrillar deposits and disease do not correlate, besides the oligomer theory, could be that only some of the possible fibril morphologies are actually harmful. It has, for example, been shown that fibrils formed without agitation are more toxic to primary rat embryonic hippocampal neurons *in vitro* than agitation induced fibrils (Petkova *et al.*, 2005). It is important to keep in mind that the studies on fibrils are almost exclusively carried out on fibrils formed *in vitro*. Whether or not all or some of the various morphologies described actually exist in an *in vivo* situation, or are just artifacts of the experimental conditions used, still remains to be seen.

$Aβ_{1-40}$ fibrils are made up by in-register parallel β-sheets (Balbach *et al.*, 2002; Petkova *et al.*, 2002) but fibrils grown with gentle agitation differ morphologically and in overall symmetry from fibrils grown under quiescent conditions. Two structural models have been developed using solid state NMR and electron microscopy and propose that protofilaments of agitation-induced fibrils are composed of two stacked layers of Aβ molecules whereas quiescent fibrils have three molecular layers in threefold symmetry around the fibril axis (Paravastu *et al.*, 2008; Petkova *et al.*, 2006) (Figure 6A, B). The β-strands in agitated fibrils are made up of residues 10–22 and 30-40 with a bend or loop in between. A stabilizing intermolecular salt bridge is formed between D23 and K28 and a hydrophobic cluster (L17/F19/I32/L34/V36) stabilizes the fold of a single layer. The

22

intermolecular side chain interactions are formed between the odd-numbered residues in strand β1 on the nth molecule and the even-numbered residues of strand β2 in the (n±2)th molecule. Interactions between layers are found between the side chains of I31 and M35 and the backbone of G37 and G33 (Petkova *et al.*, 2006). In quiescent fibrils, the β-strands are made up of residues 11-22 and 30-40. The D23-K28 saltbridge is not always present and the fold of a single layer is stabilized by H13/F19/I32/V36/V40 hydrophobic contacts. I31 and V39 form contacts between layers (Paravastu *et al.*, 2008).
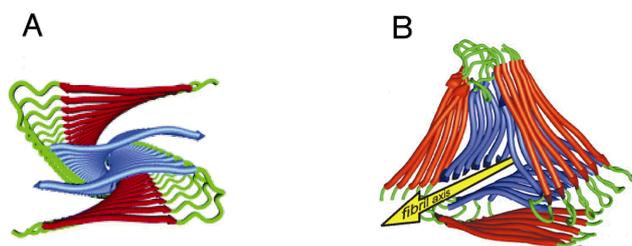

Figure 6. Proposed models for protofilament arrangement in Aβ$_{1-40}$ fibrils. A) Fibrils formed under agitation forms two-layer protofilaments. From (Petkova *et al.*, 2006). B) Fibrils formed under quiescent conditions show a three-fold symmetry around the fibril-axis. From (Paravastu *et al.*, 2008) with permission from the publisher.

Aβ$_{1-42}$ fibrils are also composed of in-register parallel β-sheets (Antzutkin *et al.*, 2002) and resemble agitated Aβ$_{1-40}$ fibrils. A recent model for Aβ$_{1-42}$(M35ox) fibrils however, indicates differences compared to the one proposed for Aβ$_{1-40}$ fibrils (Luhrs *et al.*, 2005). In this model, residues 1-17 are disordered and the β-strands are made up of residues 18-26 and 31-42. The intermolecular saltbridge between D23 and K28 is present but all intermolecular side chain interactions are formed between the odd-numbered residues in strand β1 on the nth molecule and the even-numbered residues of strand β2 in the (n-1)th molecule (Figure 7). This conformation, like that of Aβ$_{1-40}$ fibrils, would leave partially unpaired β-strands at each end of the fibril which could explain the sequence-specific co-operative mechanism of fibril extension. Another difference compared to models of Aβ$_{1-40}$ fibrils is that the protofilaments of Aβ$_{1-42}$ fibrils consist of a single layer of molecules.
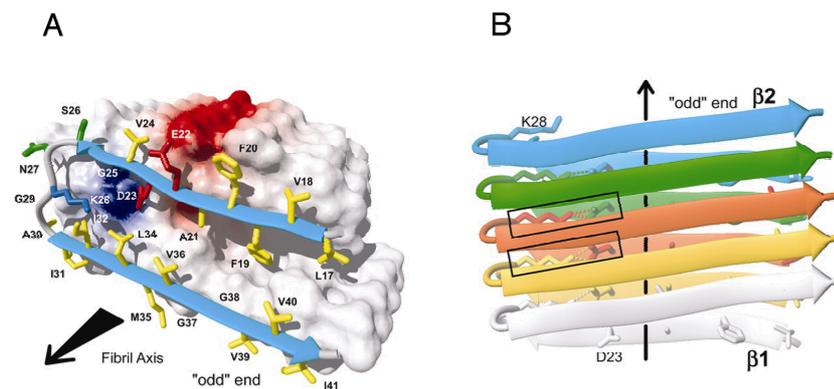
Figure 7. Model of Aβ$_{1-42}$ fibril indicating the amino acid residues making up the β–strands (A) and the orientation and offset of the strands in the fibril (B). From (Luhrs *et al.*, 2005) with permission from the publisher.

Recently, focus has been shifted from mature fibrils to soluble oligomers and early aggregates. These smaller aggregates are now believed to be the more toxic species which could explain the weak correlation between fibrillar deposits and severity of disease (Terry *et al.*, 1991). A number of different soluble assemblies, both on- and off-pathway, have been described ranging from dimers to larger aggregates of a few hundred kDa. There are few molecular structures of these aggregates, two published structures indicate β-sheet content but with different orientation of the strands (Yu *et al.*, 2009; Chimon *et al.*, 2007), which make them hard to classify and characterize. It is also possible that the choice of analytical method used for the identification can influence the structure of the oligomers. Hence, it is possible that some of the oligomers described in the literature are in fact variants of the same assembly. The study of aggregation *in vitro* is also associated with differences in protocols for solubilizing the Aβ peptide as well as small variations between different batches of synthetic peptide. Taken together all these factors further complicate the study of Aβ and its role in AD.

Protofibrils are the largest oligomers described and considered to be true intermediates in the assembly of Aβ fibrils, since they go on to form mature fibrils and can dissociate into monomers. They are shorter (< 200 nm) and somewhat thinner than mature fibrils and have been shown to be neurotoxic and to interfere with electrophysiological mechanisms associated with memory (Hartley *et al.*, 1999; Walsh *et al.*, 1999; Harper *et al.*, 1997; Walsh

24

et al., 1997). Other, non–fibrillar, soluble oligomers have been identified by several groups.

Amyloid–β–derived diffusible ligands (ADDLs) were first reported on by Oda *et al.* when they found small Aβ complexes in solutions of Aβ$_{1-42}$ co-incubated with ApoJ (clusterin) (Oda *et al.*, 1995). These complexes were thereafter shown by Lambert *et al.* to be low molecular weight oligomers around 17-27 kDa that where neurotoxic and could inhibit long term potentiation (LTP) in rat hipoocampal slices (Lambert *et al.*, 1998). A recent study combining analytical ultracentrifugation with size-exclusion chromatography (SEC), indicated that this might in fact be a more heterogenous group than first proposed with sizes ranging from monomers up to 200 kDa (Hepler *et al.*, 2006). ADDLs have been isolated from AD-affected brains and found to be present in higher levels in disease brains compared to controls (Gong *et al.*, 2003). Globulomers, Aβ$_{1-42}$ 12-mers possibly arranged in a micelle–like manner with the C-terminals of the peptides buried in the middle, are another reported oligomer that can block LTP. They have been found in brains of AD-patients and can also be prepared *in vitro* in the presence of SDS where they form independently from fibrils (Gellermann *et al.*, 2008; Barghorn *et al.*, 2005). Recently much attention was given a soluble SDS–stable 56-kDa Aβ assembly named Aβ⋆56 (Lesne *et al.*, 2006). This oligomer was isolated from brains of APP transgenic mice and found to be the cause of long term memory impairment. This negative effect on memory was also evident when Aβ⋆56 was administered to healthy, young rats. Annular Aβ assemblies have been found in *in vitro* experiments and in cell culture and have led to the channel hypothesis, suggesting that Aβ may form pore–like membrane–disrupting species (Quist *et al.*, 2005; Lashuel *et al.*, 2002).

Low–*n* SDS–resistant oligomers, dimers up to tetramers, can be secreted by cultured cells and have been isolated from human brain and cerebrospinal fluid (Walsh *et al.*, 2000; McLean *et al.*, 1999; Podlisny *et al.*, 1995). When injected into rat brains they inhibit LTP (Walsh *et al.*, 2002). Recently, Aβ dimers were extracted from human AD brains (Shankar *et al.*, 2008) and shown to inhibit LTP in mice hippocampal slices as well as to impair memory of healthy rats. Together these data further support the emerging picture of small, soluble assemblies as main synaptotoxic agents.

Since the aggregation of Aβ is a dynamic process it is likely that the population of different assemblies will vary from time to time both *in vitro*

and *in vivo*. This will further complicate the issue of determining which the true toxic species are. A large number of the described oligomers are generated *in vitro* and how all these different oligomers correlate to the *in vivo* situation is still unknown.

1.3.5 Model systems for Alzheimer´s disease *in vivo*

There is a wide gap between the numerous *in vitro* studies on the aggregation and toxicity of Aβ and the actual pathogenesis of AD. In order to overcome this problem a number of AD animal models have been created to gain better understanding of the underlying mechanisms and possible treatments. None of these models can correctly mirror all characteristics of the disease observed in humans, but they are valuable tools that allows the study of specific aspects of the disease. The most common model animal is the mouse and there are several established transgenic mouse models available (Gotz & Ittner, 2008; Woodruff-Pak, 2008). The most common ones express human APP with one or more mutations, such as the Swedish mutation (K670N/M671L, Tg2576 mice) which causes overproduction of Aβ by increased β-secretase cleavage. Secretase models in which γ-secretase activity is altered, favoring production of $Aβ_{1-42}$, are also used. The model that closest reflects human AD pathology, so far, is the 3xtg-AD mouse that expresses mutant human APP, tau and presenilin 1 (Oddo *et al.*, 2003). These mice show plaque formation, neuronal loss, memory impairment and the formation of neurofibrillar tangles.

Invertebrate models, using the nematode *Caenorhabditis elegans* and particularly the fruit fly *Drosophila melanogaster*, have emerged as powerful tools to explore the mechanisms of neurodegenerative disease and for drug screening (Link, 2005). Almost 70% of human disease related genes have homologues in *Drosophila* (Reiter *et al.*, 2001; Fortini *et al.*, 2000), 10% of which are involved in neurological disease (Greenspan & Dierick, 2004). Even though fruit flies have different neuroanatomical organization, the basic cell biology and the major neurotransmitter systems are well conserved (Iijima & Iijima-Ando, 2008), making them useful for modeling molecular and cellular processes underlying AD. Several groups have described AD models using *D. melanogaster*. The flies have a neuron-specific APP homolog (Appl) which however lacks the Aβ region, but a functional γ-secretase homolog exists. Some groups express human Aβ (1-40 or 1-42) in the CNS and retina of the flies (Crowther *et al.*, 2005; Finelli *et al.*, 2004; Greeve *et al.*, 2004; Iijima *et al.*, 2004) whereas others (Greeve *et al.*, 2004) have expressed human APP together with BACE1 to study APP processing, in

addition to Aβ toxicity. These models all exhibit reduced lifespan, neurodegeneration and behavioral deficits correlated to Aβ aggregation. Thioflavin S positive, amyloid plaque formation was seen in flies expressing $Aβ_{1-42}$. The short lifespan and short generation times of *D. melanogaster* together with their low cost also make these model systems useful for large drug screens (Crowther *et al.*, 2006).

1.3   Amyloid prevention strategies in Alzheimer′s disease

Protein misfolding diseases for which there are no cures, e.g. Alzheimer's disease, is a growing problem and finding therapeutic strategies to prevent or treat these disorders is a challenging and important task. A lot of efforts have been made in this field and the current strategies include (i) decreasing production of the amyloidogenic protein/peptide, (ii) preventing or altering aggregation or misfolding using low molecular weight compounds and (iii) neutralizing or removing misfolded, toxic species. These seemingly straightforward approaches have so far not been very successful.

In the case of AD, no effective therapies have emerged even though a large number are currently in clinical trials. There are today no available treatments that modify the disease progression, only symptomatic treatments. One example of a cognitive enhancer used to treat dementia symptoms are the acetylcholinesterase inhibitors. These compounds increase the levels of the neurotransmitter acetylcholine in synapses but do not affect any underlying cause of disease. Another available form of treatment is the *N*-methyl-D-aspartate (NMDA) receptor antagonist, memantine (Chen & Lipton, 2006). Some of the efforts in finding AD therapeutics are briefly summarized below.

1.3.1 Targeting Aβ production

Aggregation of Aβ is concentration dependent, so one possible way of targeting AD pathogenesis would be to inhibit β– or γ-secretase and thereby reduce Aβ formation. β–Secretase seems a promising target since it is the rate-limiting enzyme in Aβ production, its structure has been solved (Hong *et al.*, 2000) and it seems to have a more narrow substrate specificity than γ-secretase. However, the active site of the protease has proved to be very large, making inhibitor design challenging and very few reports on inhibitors that bind and reduce β-secretase activity exist (Ghosh *et al.*, 2008; Hussain *et al.*, 2007). γ-Secretase inhibitors that reduce Aβ synthesis have been

identified and are being tested in humans (e.g. (Fleisher *et al.*, 2008)). A problem with this approach would of course be the of β- and γ-secretase in other cellular processes. γ-Secretase has numerous substrates and cleaves for example the Notch receptor (Hartmann *et al.*, 2001), important for cell differentiation, and β-secretase has been suggested to be important for nerve myelination (Hu *et al.*, 2006). Consequently, finding ways of altering the activity of these enzymes rather than completely blocking them could prove more beneficial. Substrate-targeting γ-secretase modulators, e.g. some non-steriod anti-inflammatory drugs (NSAIDs), have been shown to affect the production of Aβ by binding to APP in the Aβ region and thereby modulate γ-secretase cleavage and the levels of released $A\beta_{1-42}$ (Kukar & Golde, 2008; Kukar *et al.*, 2008). This approach does not affect the activity of γ-secretase on other substrates, but is only applicable provided that APP or its cleavage products are not essential for any cellular functions.

1.3.2 Amyloid clearance - immunotherapy

One strategy that has been explored for Alzheimer´s disease, and that could prove to be beneficial both for prevention and treatment, is the concept of immunotherapy. Schenk *et al* (Schenk *et al.*, 1999) introduced the idea by showing that immunization with $A\beta_{1-42}$ could prevent amyloid formation in brains of young mice and also reduce the plaque burden in older mice that already had developed AD pathology. This finding was confirmed by several studies and it was also shown that passive immunization had equally positive effects (Bard *et al.*, 2000). A human trial was initiated using an $A\beta_{1-42}$ immunization protocol (Bayer *et al.*, 2005) but was terminated early due to the development of meningoencephalities in 6% of the treated patients (Nicoll *et al.*, 2003; Orgogozo *et al.*, 2003). The results of the few post mortem studies available on immunized patients show a reduction of plaques compared to the control group (Nicoll *et al.*, 2006). Currently, a number of new trials with hopefully safer vaccines are ongoing using both passive and active immunization (Nitsch & Hock, 2008). The mechanisms behind the effects seen with Aβ antibodies are unclear but proposed modes of action include the sink hypothesis (DeMattos *et al.*, 2001), where the antibody in plasma is believed to extract Aβ from the brain, as well as the idea that the antibodies will pass into the brain and aid in the clearance of plaques by for example inducing microglial-mediated phagocytosis (Schenk *et al.*, 1999).

1.3.3 Inhibition of misfolding and fibril formation

Chaperones

Molecular chaperones provide a first line of defense against protein misfolding and self-assembly in the cell, as described above in section 1.1. To utilize this protection system by enhancing chaperone activity could be a useful therapeutic approach. A number of Hsps, such as Hsp90 and the combination of Hsp 70 and 40, have been shown to effect $A\beta_{1-42}$ fibril formation at an early stage (Evans *et al.*, 2006). Hsps play important roles also in other misfolding disorders, e.g. Hsp70 has effect on α-synuclein aggregation (Dedmon *et al.*, 2005). Hsp104 suppresses fibril formation of $A\beta_{1-42}$ by interacting with various species on the fibrillization pathway and also has an amyloid-disaggregation ability *in vitro* towards Sup35-fibrils (Arimon *et al.*, 2008; Shorter & Lindquist, 2004).

   The Hsps function intracellularly but an extracellular chaperone, apolipoprotein J also called clusterin, that inhibits amorphous aggregation of various proteins has been found to inhibit fibril formation of a number of proteins such as $A\beta$ and α-synuclein (Yerbury *et al.*, 2007; Humphreys *et al.*, 1999; Matsubara *et al.*, 1996). It was proposed that the chaperone interacts with structural features common to pre-fibrillar species.

   Up-regulation of molecular chaperones has proved possible using a pharmacological approach. For example, an Hsp90 inhibitor has been found to up-regulate Hsp70 and suppress $A\beta$-induced neurodegeneration in a cell model (Ansar *et al.*, 2007). A delicate balance of chaperones will be required for beneficial effects. So far, no effective treatment utilizing this natural defense has been found.

Low molecular weight inhibitors

Small organic molecules have been found to interfere with the aggregation of $A\beta$. Some of the inhibitor compounds reported on include: Congo red, curcumin, melatonin, nicotine, rifampicin, methylene blue, scyllo-inositol, indole derivatives, RS-0406 and β-cyclodextrin (Necula *et al.*, 2007; Cohen *et al.*, 2006; Yang *et al.*, 2005; Nakagami *et al.*, 2002; McLaurin *et al.*, 2000; Pappolla *et al.*, 1998; Podlisny *et al.*, 1998; Salomon *et al.*, 1996; Tomiyama *et al.*, 1996; Camilleri *et al.*, 1994). These compounds are not similar in structure, though they are often aromatic and/or hydrophobic, and probably act in different ways and at different stages of aggregation. For example, nicotine was reported to reduce fibril formation by interacting with and possibly stabilizing the helical structure of $A\beta$ (Salomon *et al.*, 1996). RS–0406 was found in a screen of > 100,000 compounds and shown to inhibit

fibril formation, reduce Aβ–induced cell toxicity as well as rescue long term potentiation (Nakagami *et al.*, 2002). Scyllo-inositol, a naturally occurring molecule, has been shown to alter $A\beta_{1-42}$ aggregation, possibly by binding to low molecular weight oligomers and is being tested in clinical trials (Townsend *et al.*, 2006; McLaurin *et al.*, 2000). When administered to AD transgenic mice the compound improved cognition and reduced soluble levels of both $A\beta_{1-40}$ and $A\beta_{1-42}$ as well as Aβ depositions in the brain (McLaurin *et al.*, 2006).

One small-molecule inhibitory approach has been to target agents suggested to interact with Aβ and to promote fibril formation. Glycosaminoglycans (GAGs) have been implicated as mediators of Aβ fibril formation (McLaurin *et al.*, 1999). Amino-propane-sulfonic acid, APS, also called tramiposate, was designed to compete with GAGs in their binding to Aβ. This compound prevents Aβ aggregation and is currently in phase III clinical trials (Barten & Albright, 2008; Gervais *et al.*, 2007; Gervais *et al.*, 2001). Metal ions have also been suggested to be crucial for the formation of fibrils and molecular chelators that would perturb Aβ-metal binding, like clioquinol, have been tested (Cherny *et al.*, 2001).

Small molecules can be good drug candidates since they are often orally available and can cross the blood-brain barrier. However, a disadvantage with being small could be a lack of specificity. Being small only allows these inhibitors to recognize and bind limited sized regions that might not be found exclusively in the target molecule. This could make them prone to interact with various molecules. Another potential problem, especially with compounds found by screening of chemical libraries, could be that they have an unknown mechanism of action making it more difficult to optimize the structure.

Peptide-based inhibitors

In order to improve specificity, inhibitors based on Aβ segments have been developed. Tjernberg *et al.* were the first to show that the KLVFF motif ($A\beta_{16-20}$) could acts as an inhibitor of full length Aβ aggregation (Tjernberg *et al.*, 1996). Soto *et al.* developed β-sheet breaker peptides based on the segment Leu17-Ala21 of Aβ. They introduced proline-substitutions, that would reduce β-sheet propensity of the ligand and interfere with backbone hydrogen bonding, and added a charged amino acid at the end to increase solubility (Soto *et al.*, 1996). These β-breakers were shown to prevent fibril

formation, to be stable *in vivo* and to penetrate the blood-brain barrier (Poduslo *et al.*, 1999). One shorter variant (LPFFD, iAβ5) was also shown to clear amyloid plaques in a rat model (Soto *et al.*, 1998). When protected at both ends to avoid degradation, iAβ5 reduced cerebral damage and amyloid deposition in AD transgenic mice (Permanne *et al.*, 2002). Several groups have studied the effect of introducing N-methylated amino acids in peptide inhibitors based on the Aβ core region. These peptides form extended conformations and when bound to Aβ, the N-methyl substituents prevent further inter-strand hydrogen bonding and fibril elongation (Kokkoni *et al.*, 2006; Zhang *et al.*, 2006; Gordon *et al.*, 2002; Gordon *et al.*, 2001). One problem with these approaches is that they often target the later stages of aggregation, possibly stabilizing the toxic early aggregates. Recently however, Austen *et al.* have demonstrated the design of peptide inhibitors based on the $Aβ_{16-21}$ sequence, targeting oligomeric species instead (Austen *et al.*, 2008). There have also been attempts to use C-terminal derived peptides of $Aβ_{1-42}$ as potential inhibitors of oligomer formation. Fradinger *et al.* have shown that the Aβ-fragments 31-42 and to some extent 39-42 potently disrupt oligomer assembly of $Aβ_{1-42}$ and are able to reduce Aβ-induced cell toxicity (Fradinger *et al.*, 2008). Another peptide-based inhibitor of oligomerization, combining the β-breaker strategy with inhibition of aromatic recognition, was recently described (Frydman-Marom *et al.*, 2009). This small molecule, D-Trp-Aib, was shown to be orally available, inhibit Aβ toxicity towards cells and to improve cognitive performance and reduce the plaque load in AD mice.

1.4 Surfactant protein C

Human lung surfactant protein C (SP-C) is a highly hydrophobic transmembrane peptide consisting of 35 amino acids residues (Beers & Lomax, 1995). It is expressed exclusively in alveolar type II epithelial cells and is secreted, together with phospholipids and SP-B, into the alveoli where it aids in lowering the surface tension at the air-water interface, thereby preventing alveolar collapse at end expiration (Whitsett & Weaver, 2002).
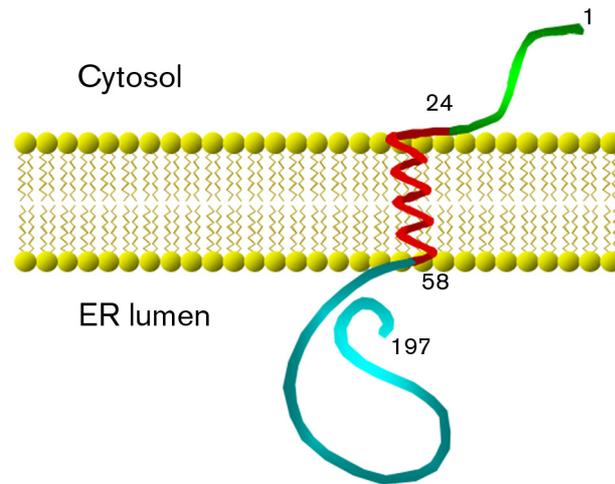
Figure 8. Schematic view of proSP-C situated in the ER membrane. The N-terminal (green) corresponds to residues 1-23 and resides in the cytosol. Residues 24-58 make up the mature SP-C (red) and residues 59-197 corresponds to the C-terminal (blue) located in the ER lumen. (Figure from Prof. Timothy Weaver)

SP-C is expressed as a 197-residue integral membrane proprotein (proSP-C) which is anchored in the endoplasmic reticulum (ER) membrane in a type II orientation, i.e. with the C-terminal in the ER lumen (Figure 8). The mature peptide is found in the transmembrane region and corresponds to residues 24-58. The N-terminal propeptide (residues 1-23) is important for the trafficking and stability of the proprotein in the secretory pathway (Li *et al.*, 2004; Conkright *et al.*, 2001) whereas the C-terminal domain, CTC (residues 59-197), has an unknown function. Expression of proSP-C, lacking parts of the C-terminal, results in ER stress, intracellular protein aggregation and cytotoxicity which indicate that this region is somehow vital for the stability of the proprotein (Mulugeta *et al.*, 2005; Bridges *et al.*, 2003; Kabore *et al.*, 2001).

Mutations in the SP-C gene are associated with interstitial lung disease and cause the protein to misfold and aggregate intracellularly (Nogee *et al.*, 2002; Thomas *et al.*, 2002; Nogee *et al.*, 2001). Most disease-linked mutations found in the proSP-C gene are located in CTC, and specifically in a domain known as Brichos, covering residues 94-197 (Beers & Mulugeta, 2005). Brichos is a novel domain, consisting of around 100 amino acid residues, that has been found in over 100 proteins divided into 11 families, based on sequence similarity (Hedlund, 2009). These previously

unrelated proteins are associated with various functions and some degenerative and proliferative disorders e.g. ITM2B (Bri) associated with amyloid formation in familial British and Danish dementia (Kim *et al.*, 2000; Vidal *et al.*, 2000). The proteins in the Brichos families are all made up of four regions, organized in the same manner, starting with a hydrophobic stretch followed by a linker region, the Brichos domain and finally a C-terminal part. The only exception to this structure is proSP-C which lacks a C-terminal extension after the Brichos part. Only three residues are strictly conserved in the domain; one aspartic acid and two cysteine residues. In recombinant CTC, these cysteines form a disulfide bridge (Casals *et al.*, 2008). Since the Brichos domain clearly exist in various proteins, numerous functions have been proposed for it, including a role in post-translational processing and a chaperone-like function (Sanchez-Pulido *et al.*, 2002). However, up till now there has been no experimental support for such functions.

The transmembrane α-helix of SP-C is discordant, i.e. it is composed of amino acids with a high propensity to form a β-strand, mostly Val and Ile (Kallberg *et al.*, 2001). These amino acids are branched at the β-carbon which disfavors α-helix formation, making the helix less efficient in folding. For this reason, although stable once folded and inserted into the membrane, the region is unstable in solution and when unfolded it doesn't retain its structure but will spontaneously convert to β-sheet aggregates and to amyloid-like fibrils (Szyperski *et al.*, 1998). Fibrils of SP-C have been isolated from patients with the lung disease pulmonary alveolar proteinosis, adding SP-C to the list of proteins capable of forming amyloid-like fibrils (Gustafsson *et al.*, 1999).

2 Present investigation

2.1 Scope of this thesis

The overall aim of this thesis was to study ways of affecting protein misfolding and subsequent formation of amyloid-like fibrils, with emphasis on Aβ.

In particular we performed studies aimed to:

➢ Investigate the possibility to stabilize the helical structure of Aβ, using small designed ligands, in order to prevent amyloid fibril formation and toxicity (**paper I–II**).

➢ Elucidate the role of CTC, and its Brichos domain, in the folding and stabilization of proSP-C and mature SP-C (**paper III**).

➢ Characterize the binding specificity of CTC and investigate its potential role as a general anti-amyloid chaperone (**paper IV–V**).

2.2 Methods

In the following section some of the methods used in this thesis are summarized. Detailed descriptions of the experimental procedures can be found in the respective papers

2.2.1 Transmission electron microscopy

Transmission electron microscopy (TEM) is a powerful technique based on the same principles as light microscopy that uses electrons instead of light. The resolution of light microscopy is limited by the wavelength of light but by using a focused beam of electrons, with lower wavelength, the resolution can be improved around a thousand times, yielding a magnification up to 500,000-1,000,000 times. Briefly, a beam of electrons is passed through the specimen of interest and the interactions with the molecules in the sample will cause some of the electrons to scatter. The unscattered part of the beam will create patterns on the detection plate that correspond to the densities of various parts of the sample where darker areas indicate electron dense parts.

For TEM analysis the sample needs to be thin enough to allow the electrons to pass and contain molecules that diffract electrons. Biological samples are composed mainly of atoms of low electron density (e.g. carbon, hydrogen and nitrogen) and will therefore allow most electrons to pass undisturbed, resulting in poor contrast. In order to improve contrast, negative staining of the samples can be employed. The stains used are usually heavy metal salts, e.g. uranyl acetate, which will easily interact with the electron beam. The stain will not bind to the sample but will instead surround it so that the edges of the specimen will absorb most electrons – in the end creating a "negative" image.

2.2.2 Circular dichroism spectroscopy

Circular dichroism (CD) spectroscopy is a technique that makes use of the fact that chiral molecules will interact differently with right- and left-circularly polarized light (Woody, 1995). Peptides and proteins have chiral properties and contain a number of groups with characteristic absorption patterns, chromophores, with the main chromophore in far-UV CD (< 250nm) being the amide group in the peptide bond.

The sample to be analyzed is illuminated with plane polarized light, which consists of right- and left-circularly polarized beams of light with the

same intensity (Figure 9A). The chiral molecules will absorb the two beams to different extent and due to this phenomenon the outgoing light will be converted into elliptically polarized light (Figure 9B).
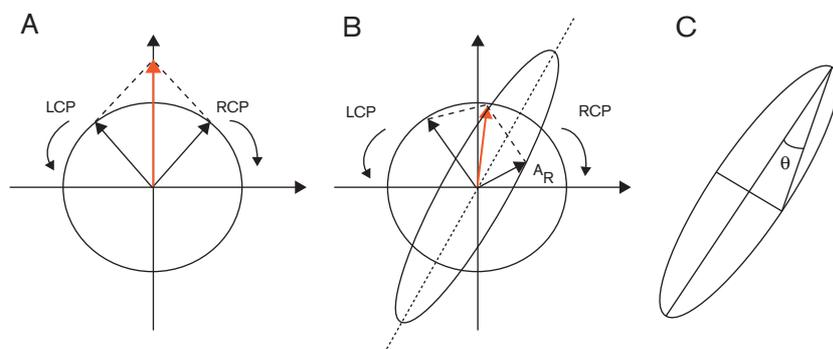

Figure 9. Circular dichroism spectroscopy. A) The light reaching the sample consist of two circularly polarized light-beams with different rotations (left and right circularly polarized) – making up the plane polarized beam (red arrow). B) When the light passes through the sample the two components will be absorbed differently and the outgoing light will be elliptically polarized (red arrow). C) Ellipticity (θ) is defined as the indicated angle in the resulting ellipse and describes the degree of circular dichroism.

CD is defined as the difference in absorption (A) of right (R) and left (L) circularly polarized light

$$CD(\lambda) = A_L(\lambda) - A_R(\lambda)$$

Ellipticity, defined as the angle θ (Figure 9C), is a normal quantity used for describing the degree of circular dichroism. θ can be related to ΔA according to:

$$\theta = 32.98 \, (A_L - A_R)$$

Usually spectra are reported using mean residual molar ellipticity ($[\theta]$, unit: kdeg x cm^2 x dmol^{-1}) in order to remove the dependence of path length (l), concentration (c) and number of amino acid residues (n):

$$[\theta] = 100\theta/(c \cdot n \cdot l)$$

37

CD can be used for estimation of secondary structure elements as well as for monitoring conformational changes, e.g. from denaturation or ligand binding. Different secondary structure elements give rise to specific patterns when the molar ellipticity is plotted against wavelength. Figure 10 summarizes the characteristics of CD spectra obtained from proteins with different secondary structures.
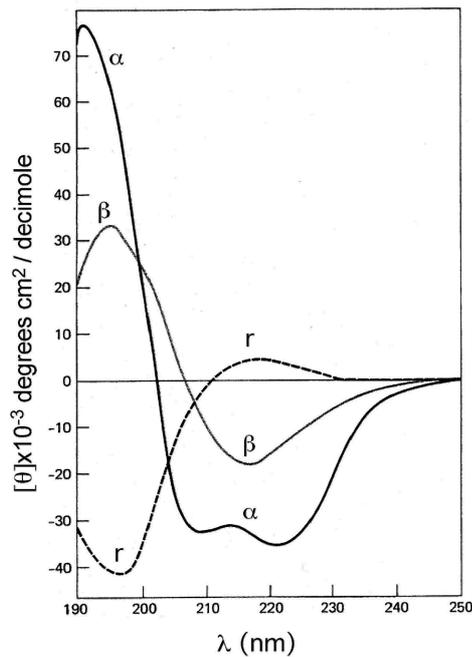
Figure 10. Characteristic circular dichroism spectra of proteins with α-helical (α), β-sheet (β) or random-coil (r) structures.

2.2.3 Mass spectrometry

Mass spectrometry (MS) is a technique for measuring the mass of a molecule, that can be used for characterizing proteins and peptides. The outline of a MS instrument is briefly as follows; the sample of interest is first introduced, ionized and transferred into the gas-phase in an *ion source*, the produced ions are separated in vacuum according to their mass-to-charge ratio (m/z) in a *mass analyzer* and are thereafter detected in a *detector* (Edmond de Hoffman, 2002).

The problems associated with transferring large biomolecules, such as proteins and peptides, into the gas-phase were resolved when two soft-ionizations techniques were introduced in 1987-1989: matrix-assisted laser desorption/ionization (MALDI) (Karas & Hillenkamp, 1988) and electrospray ionization (ESI)(Fenn *et al.*, 1989). Using these techniques, briefly described below, fragmentation can be avoided and multiple charges can be imposed on molecules which will lower the m/z ratio making them easier to detect.

Matrix-assisted laser desorption ionization

In the MALDI process the sample is first co-crystallized with a light-absorbing solution, often a weak aromatic acid, called a matrix. The formed crystals containing both sample and matrix are thereafter irradiated with a laser. The matrix molecules will absorb the energy of the laser resulting in vaporization and expansion of the matrix into the gas-phase taking the sample molecules with it. In the gas-phase, protons are transferred from the matrix molecules to the sample creating ions that can be separated and detected. This technique yields mainly singly-charged ions and few fragments. It is compatible with non-volatile solvents and is less sensitive to contaminating compounds compared to ESI.

Electrospray ionization

In ESI the sample solution is pumped through a capillary over which a strong electric filed is applied, creating a "spray" of charged droplets at the tip of the capillary. On the way to the mass spectrometer the solvent evaporates from the droplets creating a cascade of ruptures due to the charge concentration, yielding even smaller highly charged droplets. This process will finally result in the release of charged analyte molecules that can then be analyzed. The analyte molecules generally receive their charge through gas-phase proton-transfer. This technique requires the use of volatile solvents, results in very little fragmentation and yields multiple-charged ions, making detection of large molecules easier.

2.2.4 Fluorescence spectroscopy

Fluorescence is a form of luminescence, the emission of photons from electronically excited states. It utilizes the fact that some molecules, fluorophores, can be excited from their ground state to a higher energy state,

called an excited state, via absorption of light. This higher energy state is unstable and when the molecule returns to the ground state again a photon with a different wavelength (lower energy) is emitted and can be measured (Lakowicz, 1983).

Intrinsic fluorescence

In proteins and peptides the aromatic side chains of tyrosine, tryptophan and phenylalanine are intrinsic fluorophores. Of the three, tryptophan exhibits the highest fluorescent yield whereas the other two are weaker emitters in comparison. The emission spectra of proteins are sensitive to binding of substrates, protein-protein interactions and denaturation and changes in intrinsic fluorescence can be used to study structural changes (Lakowicz, 1983). In this thesis Trp-fluorescence was employed to study the ligand-Aβ interactions with the Trp-containing ligand used in paper II. For this purpose exitation at 280 nm was used and emission was observed between 290 and 400, with a peak at 348 nm.

Thioflavin T fluorescence

Thioflavin T (ThT), a cationic benzothiazole dye, is widely used for the detection of amyloid fibrils both in solution and in tissue. Upon binding to amyloid fibrils, this dye exhibits an enhanced fluorescence with an emission maximum at 482 nm and a new absorption peak at 450 nm (LeVine, 1993). The mechanism behind this phenomenon and the exact structure that ThT binds to is not clear. It has been suggested that the dye, with a hydrophobic part linked to the polar benzothiazole group (Figure 11), could form micelles in aqueous solution and that binding of these micelles to the fibrils would cause the change in fluorescence (Khurana *et al.*, 2005). Binding of monomeric ThT to channels within the β-sheet of the fibrils has also been suggested (Krebs *et al.*, 2005).
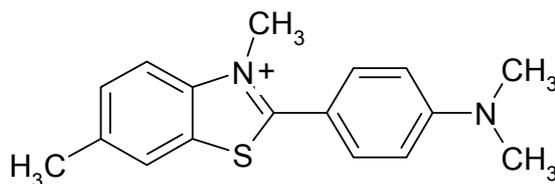
Figure 11. Molecular structure of Thioflavin T.

2.2.5 Model systems

Drosophila melanogaster model

For **paper I** a *Drosophila melanogaster* model of $A\beta_{1-42}$ aggregation generated by Crowther *et al.* (Crowther *et al.*, 2005) was used. This transgenic strain express two gene copies coding for human $A\beta1-42$, one on chromosome 2 and one on chromosome 3. The genes code for $A\beta_{1-42}$ coupled with a secretion signal peptide to direct the synthesis of $A\beta$ to the ER. The UAS/Gal4 activation system (Brand & Perrimon, 1993) was used in order to control gene expression. The final transgenic strain was obtained by crossing transgenic $A\beta$ flies with flies transgenic for the Gal4-elav^{c155} pan-neuronal driver (Lin & Goodman, 1994), which will drive $A\beta_{1-42}$ production in the CNS and retina of the fly. The expression and accumulation $A\beta$ in the transgenic flies resulted in an observed reduction of locomotor activity as well as a reduced lifespan compared to non-transgenic flies.

Cell models

For this thesis two different cell models were used. For the aggregation studies in paper **III** human embryonic kidney cells (HEK293) were used and for the toxicity studies in paper **I** and **II** we used PC12 cells. HEK293 is a commonly used cell line generated by transformation of a human embryonic kidney cell line with DNA from human adenovirus type 5 (Graham *et al.*, 1977). PC12 is a cell line derived from a rat pheochromocytoma, a tumor of the adrenal medulla, which has a neuroectodermal origin (Greene & Tischler, 1976). PC12 cells are often used as a model system in neurobiology and neurochemistry and will, when grown in the presence of nerve growth factor (NGF) extend neurites and increase the synthesis of a number of neurotransmitters – similar to neuronal differentiation. However, in paper **I** and **II**, undifferentiated PC12 cells were used since these cells are known to be sensitive to $A\beta$ induced toxicity.

2.3 Results and discussion

2.3.1 α-Helix targeting reduces amyloid β-peptide aggregation and toxicity

There is no consensus in the AD field as to which intermediate species on or off the fibrillation pathway that are truly neurotoxic. However, it is likely that misassembly of Aβ play a role in the disease. From this perspective targeting intermediates might result in the accumulation of toxic assemblies. In light of this, we set out to find a way of stabilizing the native monomeric structure of Aβ, thereby preventing misfolding and aggregation. This strategy has been employed for other aggregation prone proteins such as superoxide dismutase (SOD) (Ray *et al.*, 2005; Ray & Lansbury, 2004) and the amyloidogenic transthyretin (Hammarstrom *et al.*, 2003; Miroy *et al.*, 1996) where stabilization of a native dimer or tetramer, respectively, prevented dissociation into monomers and aggregation. The approach, termed native state stabilization, means that the folded state is selectively stabilized over the un/misfolded state, which will raise the barrier for unfolding.

It has been argued that the concept of native state stabilization would not be applicable to intrinsically disordered peptides and proteins like Aβ. However, Aβ shows secondary structure propensities in its C-terminal and middle regions, especially in stabilizing environments like membranes. It contains a discordant helix covering residues 16–23 and this helical structure probably needs additional support when it loses its stabilizing interactions after release from the membrane environment (Paivio *et al.*, 2004; Kallberg *et al.*, 2001). It has recently been proposed that stabilization of helical structures in $Aβ_{1-40}$ as well as $Aβ_{1-42}$ would block oligomerization by preventing the conversion into β-structure (Yang & Teplow, 2008).

Using molecular modeling two classes of molecules, targeting and stabilizing the discordant helix of Aβ, were designed (**papers I-II**). One class included the peptoids Pep1a, Pep1b and Pep2. The other class consisted of four decanoyl polyamines; Dec-DETA, Dec-TETA, Dec-DPTA and Dec-Spermine. All of the compounds were designed to interact with hydrophobic patches and charged amino acid side chains in the 13–23 region of Aβ, residues that could potentially destabilize the helix. For structures and proposed modes of interaction, see Figure 12.
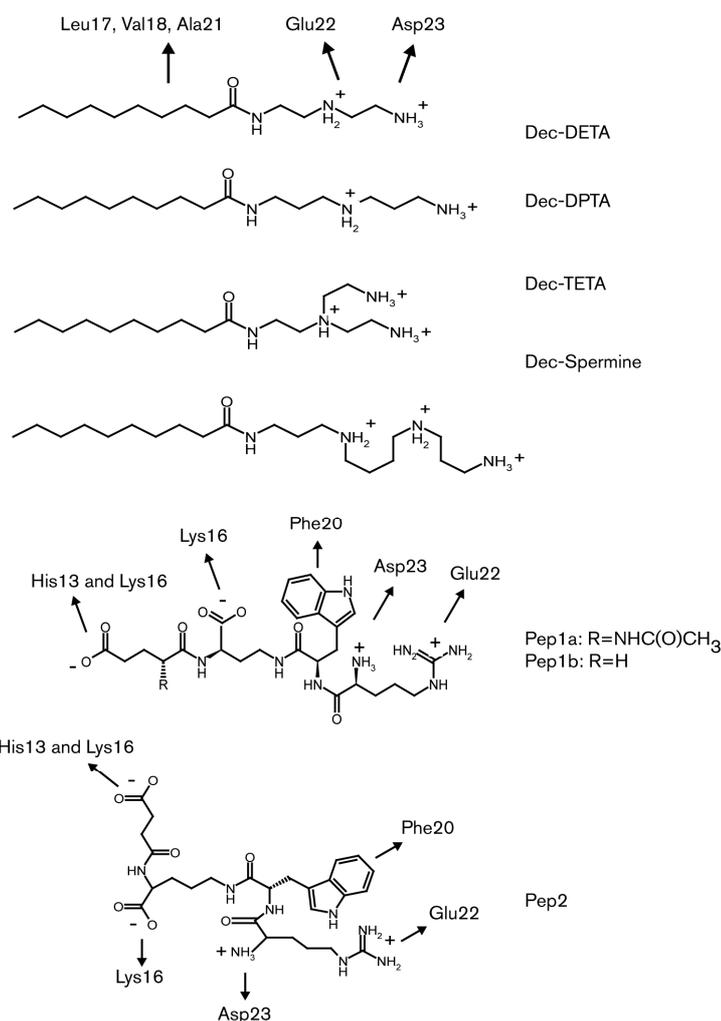
Figure 12. Structures of ligands designed to stabilize the 13-23 region of Aβ in a helical conformation. The proposed interactions are indicated.

In **paper I**, Pep1a, Pep1b and Dec–DETA were evaluated and found to potently affect the aggregation and fibril formation of $A\beta_{1-40}$ *in vitro*. The peptoids reduced fibril formation whereas Dec–DETA induced formation of shorter and thicker fibrillar species that exhibited an unexpected increase in ThT–fluorescence. All three ligands were able to stabilize a helical conformation in fragments of Aβ harboring the discordant helix. The interactions observed were sequence dependent since no structural changes were seen when the ligands were added to a scrambled version of $A\beta_{13-23}$. In

an MTT-assay, using the PC12 cell line, the ligands reduced $A\beta_{1-42}$ induced toxicity and they also had the ability to prevent reduction of γ-oscillations, associated with Aβ in mouse hippocampal slices. Measuring electrophysiological functions such as γ–oscillations is a good way of determining the toxic effect of Aβ since they are important for memory and cognition (Engel & Singer, 2001) and are markedly reduced in Alzheimer´s patients (Ribary *et al.*, 1991). To evaluate the oral availability and the potency of our ligands to affect Aβ aggregation *in vivo*, Pep1a and Dec-DETA were administered to *Drosophila melanogaster* expressing $A\beta_{1-42}$ in their CNS. An improvement in longevity and mobility, in combination with a reduction in nervous tissue destruction, was observed in the treated flies. The specificity of the ligands was tested by administering them to wild-type flies and flies expressing mutant huntingtin, an aggregation–prone protein linked to Huntingtons disease. No rescuing effect was observed in these flies, verifying the Aβ specificity of the designed compounds. Together these data suggest that stabilizing the discordant helix of Aβ with small designed ligands is a possible therapeutic strategy to prevent misfolding and toxicity.

Since the concept of native state stabilization seems to work for Aβ (**paper I**), we expanded the design and synthesis of ligands (**paper II)** to investigate how small structural changes would affect the interactions. One new peptoid, Pep2, and three additional decanoyl polyamines, Dec-TETA, Dec-DPTA and Dec-Spermine, were evaluated. Pep2 proved to be as efficient as Pep1 in preventing fibril formation, it induced helical conformation in Aβ-fragments containing the 16-23 region and bound to helical $A\beta_{13-23}$ as judged by fluorescence spectroscopy. It also reduced $A\beta_{1-42}$ induced cell toxicity in PC12 cells. However it did not reduce ThT-fluorescence as might be expected from the observed decrease in fibril formation. It is possible that Pep2 promotes the formation of Aβ-oligomers capable of ThT-binding with a reduced cell-toxicity compared to species formed in solutions of Aβ alone. The three decanoyl derivatives all stabilized a helical conformation in Aβ-fragments with specificity and reduced ThT fluorescence by 50 %. Using ESI-MS they were shown to bind full-length $A\beta_{1-40}$ and they affected fibril formation to varying degrees with Dec-Spermine being the most potent inhibitor as judged by TEM. In cell-assays all three decanoyl derivatives reduced $A\beta_{1-42}$ cell toxicity, with Dec-DPTA showing the most prominent reduction.

Small molecules targeting Aβ and preventing amyloid–like fibril formation has been reported on extensively. However most act by unknown

mechanisms and probably with low specificity. Here we show that by targeting the central helix of Aβ it is possible to generate Aβ-specific, orally available compounds with an inhibitory effect on aggregation. A problem with some known inhibitors of fibril formation, recently pointed out, is that they also function as chemical aggregators and that this is the basis of their inhibitory effect (Feng *et al.*, 2008). This, however, was shown not to be the case for these ligands.

The compounds presented in **papers I** and **II** should be seen as starting points for further optimization. The fact that small structural changes are allowed, without affecting the activity of the ligands, indicates there is a degree of freedom in designing new compounds as long as the basic requirements of the concept are met.

2.3.2 Novel chaperone with anti-amyloid properties

SP-C is a transmembrane protein, with a poly-Val helix prone to misfold, that have been shown to form amyloid-like fibrils. The protein is expressed as a proprotein, proSP-C, with a C-terminal part, CTC, which contains a Brichos domain, suggested to have a chaperone-like function. This proposed function for the Brichos domain, together with the fact that a number of mutations in CTC have been linked to interstitial lung disease lead us to investigate the function of this C-terminal propeptide.

In **paper III**, we show that CTC binds to mature SP-C only when it is in a non-helical conformation and that it has the ability of preventing SP-C from forming amyloid-like fibrils *in vitro*. To study the effect of CTC on the folding and stability of the proprotein, witldtype proSP-C and proSP-C with three different mutations linked to disease, I73T, L188Q and ΔExon4, were expressed individually in HEK293 cells. Only the mutations located in the Brichos domain, i.e. L188Q and ΔExon4, resulted in aggregation and formation of amyloid-like deposits. Co-expressing CTC (carrying an ER–retention signal) with proSP-C^{L188Q} abolished amyloid formation and it was shown that CTC binds mutant proSP-C in significantly higher degree than wildtype proSP-C. These data indicate that CTC is important for the stabilization and correct folding of the proprotein in the ER and that its activity is most likely linked to the Brichos region. Replacing the transmembrane poly-Val segment in the proprotein with a poly-Leu stretch, which has a higher α–helix propensity, stabilizes proSP-C^{L188Q} indicating that it is the transmembrane region that drives aggregation. Together these results

implicate an anti-amyloid role for CTC as an intramolecular chaperone. We propose that CTC, via the Brichos domain, stabilizes the transmembrane poly-Val region of proSP-C until it has attained its correct fold, thereby preventing aggregation and amyloid formation of this unusually hydrophobic and folding-incompetent protein. Intramolecular chaperones and pro-sequence assisted folding have been found for a number of proteins, such as subtilisin (Ikemura *et al.*, 1987) and carboxypeptidase Y (Winther & Sorensen, 1991). The chaperone activity in these is usually located to the C- or N-terminal extensions in the proprotein (Chen & Inouye, 2008). From our data it seems possible that CTC can exert its function either intramolecularly or in trans, on neighboring proteins, like a molecular chaperone.

In an attempt to further characterize the function of CTC and especially the substrate specificity and requirements for binding, a SPOT analysis was performed (**paper IV**). Using SPOT membranes with bound decamers of amino acids it was shown that CTC specifically recognises and binds to residues that promote membrane insertion, according to the "biological hydrophobicity scale" (Hessa *et al.*, 2005). Transmembrane regions need to expose nonpolar sidechains and hide their polar backbone in order to fit inside cellular membranes. Consequently, membrane insertion is promoted by the presence of the amino acids Ile, Leu, Phe and Val and also by a α-helical conformation (Hessa *et al.*, 2005). In **paper IV** it is also shown that CTC is able to distinguish between target peptides that are in different conformations, binding only to β-strand conformations – in line with previous observations (**paper III** and (Johansson *et al.*, 2006)). These data imply that CTC can recognise and bind to transmembrane segments that have not yet folded into the required α-helical conformation, acting in a chaperone-like manner. This proposed function fits well with the fact that proSP-C has a transmembrane segment, mainly consisting of Val, with an instrinsic prodisposition to misfold that needs assistance to avoid misfolding and formation of amyloid-like fibrils.

General properties of transmembrane proteins overlap to some extent with the proposed main determinants for amyloid formation, including hydrophobicity and overall charge. Initial studies (**paper IV**) showed that the anti-amyloid function of CTC found for SP-C extends to $A\beta_{1-40}$, which also contains a transmembrane helix. In **paper V** the interaction between CTC and $A\beta_{1-40}$ was further explored and the ability of CTC to act as a chaperone for amyloidogenic peptides was evaluated. It was shown that

CTC keeps $A\beta_{1-40}$ in a soluble form and potently inhibits it from aggregating and forming amyloid–like fibrils in a substoichiometric ratio, but is unable to dissociate pre–formed fibrils. However, CTC did not show classic chaperone activity, again pointing to the possibility that it is a chaperone targeting specific types of proteins only. $A\beta_{1-40}$ and CTC formed stable complexes that allowed for SEC and ESI–MS studies. Using SEC a complex of 110 kDa, possibly corresponding to a 12–mer of $A\beta_{1-40}$ together with a CTC trimer, was found. This in line with previous findings showing that CTC preferentially forms trimers in solution (Casals *et al.*, 2008). Stable 12–mers of $A\beta$ has also been reported on (Lesne *et al.*, 2006; Barghorn *et al.*, 2005). The ESI–MS experiments however, indicated that the dominating species would be a CTC trimer bound to monomeric $A\beta$. Taken together, the data suggests that complexes are formed between a CTC trimer and an $A\beta$ molecule, possibly present in a 12–mer assembly. CTC was also found to prevent the amyloidogenic protein medin from forming amyloid–like fibrils indicating that CTC might possess a more general anti–amyloid activity.

In conclusion, we propose that CTC, possibly via its Brichos domain, has the ability of acting as a chaperone, recognizing unfolded transmembrane segments and stabilizing them until they have obtained their correct fold. This function, which to our knowledge has not been observed for any other protein, probably originates from the fact that proSP–C is highly aggregation prone and in need of folding–assistance. It is also possible that this ability is coupled with a more general anti–amyloid function that could be applicable to other non–transmembrane and/or amyloidogenic proteins. Recently, extracellular chaperones have been implicated in the prevention of amyloid fibril formation. In light of this, the properties described here make CTC, and especially the Brichos domain which is found in other pro–proteins as well, interesting from a diagnostic and therapeutic perspective. Brichos domains have been found in other pro–proteins associated with amyloid formation like the Bri protein. The possibility of using this new, naturally occurring, chaperone to prevent the formation of toxic amyloid assemblies needs to be further explored.

3   Concluding remarks

Folding of proteins remains an important but incompletely understood process. Misfolding and aggregation of proteins is linked to a large number of diseases, causing problems due to loss of function or, in the case of amyloid disorders, gain of toxic function. A number of natural mechanisms that prevent misfolding and misassembly exist and include e.g. localization of charged residues in edge β-strands to prevent β-sheet formation (Richardson & Richardson, 2002) and structural gatekeepers in the form of charged residues which interrupt stretches of hydrophobic residues and thereby prevent unwanted interactions (Otzen *et al.*, 2000). In addition, molecular chaperones aid in the folding process.

Amyloid fibrils are insoluble aggregates, formed by a number of proteins and are linked to various diseases. For aggregation to occur, globular proteins most likely need to be destabilized and partially unfolded whereas disordered proteins, such as Aβ, need to obtain partial order similar to that found in the aggregates, i.e. β-strands for amyloid fibril formation. It has been suggested that Aβ can exist as a β-hairpin prior to aggregation which is in line with this assumption. The kinetics of misfolding and misassembly are likely to be of importance and small changes in the rate of aggregation could prove to be very beneficial in misfolding diseases. With this in mind, stabilization of the native state or reducing the amount of protein produced seems like a natural place to start in the search for therapeutic approaches.

In this thesis two strategies for preventing misfolding and amyloid formation are presented and evaluated. One utilizes the concept of native state stabilization with the aid of small designed molecules targeting the discordant α-helix of Aβ and the other involves a naturally occurring

chaperone with affinity for unfolded transmembrane segments, that has proved to exhibit anti-amyloid properties.

Using rationally designed compounds, to target the helical region of Aβ, will most likely improve the specificity compared to compounds found by screening of large libraries. The compounds described in this work should be viewed as support of a novel strategy of inhibition. In order to be considered as potential drug candidates, the structures need to be adjusted to further improve specificity and affinity. Protease resistance and ability to efficiently cross the blood-brain-barrier are also important characteristics that need to be considered. However, the initial studies made are encouraging.

Data on Aβ, its aggregation and the connection to Alzheimer´s disease, are confusing. Numerous oligomers and intermediates on or off the fibrillation pathway have been reported and shown to induce cell toxicity and memory impairment. To further complicate the matter there are no clear data on how Aβ exerts its toxicity, or which species that are involved in the *in vivo* situation. It cannot be ruled out that there are other key players in the disease, besides Aβ and its precursor protein, that are yet to be found. Establishing which, if any, of the soluble Aβ species observed that are actually relevant for the disease is a challenging and important task, especially for the future development of therapies. Stabilizing a monomeric peptide rather than targeting later stages of the aggregation could eliminate the risk of accumulating harmful oligomers. In a membrane environment, Aβ exhibit helical structure in two regions, that are later involved in the formation of β-sheets. The conversion from a α-helical to β-strand conformation has been proposed to be essential for fibril formation. Stabilizing helical conformation, in at least one of the two regions of Aβ forming strands in the fibrils, could prevent toxic aggregation as shown in this thesis.

Besides native structure stabilization, removal of different types of aggregates could also be a successful strategy in misfolding disorders. An approach that has the potential of accomplishing both of these tasks is the use of chaperones. It is possible that a decline in chaperone activity with age may be a major contributing cause in diseases associated with protein aggregation and deposits thereof. Studies presented in this thesis show the existence of a chaperone, CTC, which inhibits fibril formation of amyloidogenic proteins. CTC, containing a Brichos domain, has most likely evolved to prevent misfolding and subsequent aggregation of a highly

hydrophobic, folding incompetent, part of proSP-C. Unlike molecular chaperones, that display little substrate specificity, CTC recognizes elongated stretches of nonpolar amino acid residues with high specificity. If this specificity and chaperone activity apply to other Brichos domains, needs to be investigated. Solving the structure of CTC might shed more light on the mechanism by which it acts, possibly revealing the region involved in binding.

The ability of CTC to act in a chaperone-like manner towards amyloidogenic proteins is intriguing. A plausible explanation for its activity is outlined in Figure 13. Briefly, CTC recognizes elongated conformations of peptides containing primarily the amino acid residues Val, Ile, Leu and Phe. Binding to these segments will inhibit further aggregation and possibly allow for re-folding (Johansson, 2009). This function might also include an ability to bind to soluble aggregates, which expose CTC-binding epitopes, and promote their uptake and degradation by nearby cells, like extracellular chaperones. If so, this would clearly be a promising lead and deserves to be investigated further.
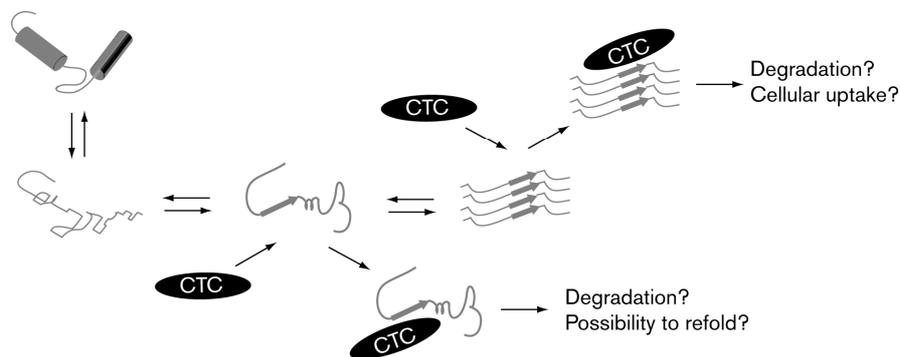
Figure 13. Proposed mode of action of CTC.

Interacting with and stabilizing native states of proteins prone to misfold is a tempting and highly conservative strategy, preventing disease associated events as early as possible. This is especially true for situations where the mechanism of toxicity is poorly understood, like the role of Aβ in Alzheimer´s disease. The two novel strategies outlined in this thesis can both target monomeric states of aggregation-prone proteins. In addition, if the

chaperone-like activity towards amyloidogenic proteins of CTC can eliminate early aggregates as well, it could be possible to use CTC as a starting point of new chaperone-based therapeutics in the field of misfolding diseases.

An interesting difference between the two types of compounds described in this thesis, α-helix targeting ligands and CTC, is their origin. CTC has evolved naturally probably due to a need for folding assistance whereas the ligands are an attempt on our behalf to control a naturally occurring event. The efficiency and applicability of the two strategies remains to be seen, but it is tempting to suggest that CTC, due to its origin, might show a better specificity and affinity than the designed ligands. However the size and the fact that it is a protein could prove problematic in terms of crossing the blood-brain-barrier and being stable in novel environments. From this perspective, small man-made ligands have a clear advantage in that they can more easily be manipulated and targeted towards specific locations. So the intriguing question remains – can we control nature better than nature itself?

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