Domain swapping as a molecular mechanism in amyloidosis

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Cover illustration

Human cystatin C can be oligomerized *in vitro* to form doughnut-shaped objects, which on further incubation yield amyloid fibrils. Both structures can be visualized by electron microscopy. The *in vitro* fibrils are a model for *in vivo* amyloid formed in amyloidosis. In this doctoral thesis it is proposed that the *in vitro* cystatin C oligomers are formed by propagated domain swapping. This figure was prepared by Karolina Michalska, Poznan University, Poznan, Poland.

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Dans les champs de l'observation

le hasard ne favorise que les esprits préparés.

Louis Pasteur (1822-1895)

Contents

1. Abstract

Amyloidosis is a group of diseases characterized by a change in protein conformation resulting in aggregation and tissue deposition of amyloid fibrils. One variant of cystatin C, L68Q cystatin C, is highly amyloidogenic and persons carrying the corresponding gene suffer from massive cerebral amyloidosis leading to brain hemorrhage and death in early adult life. Amyloidogenic proteins like cystatin C and prion proteins have been shown to form dimers by exchange of subdomains of the monomeric proteins. This process, called ”domain swapping”, has also been suggested to play a part in the generation of amyloid fibrils. In the present work the role of this mechanism is investigated. Two variants of wild type and L68Q cystatin C, respectively, with disulfide bonds at positions selected to inhibit domain swapping have been produced. It was observed that the capacity of the four variant proteins to form dimers, oligomers and fibrils was prevented, implying an important role of “domain swapping” in the aggregation process. Oligomers of different amyloidogenic proteins have been reported to precede fibril formation, suggesting oligomers as intermediates in fibrillogenesis. Indeed, oligomers of wild type cystatin C were detected already in the beginning of the lag phase of the fibrillization reaction. The appearance of these doughnut-shaped oligomers is similar to that described for *e.g.* the Aβ peptide, involved in Alzheimer disease. Purified oligomers of cystatin C were shown to fibrillize faster and at a lower concentration than the monomeric protein, suggesting a role of the oligomers as fibril-assembly intermediates. Thereafter, it was investigated whether or not species larger than dimers can be detected *in vivo*. Indeed, high molecular weight cystatin C immunoreactive components, with a molecular mass between 100 and 150 kDa, were found in blood serum and brain obtained from a transgenic mouse, expressing the human L68Q cystatin C gene, but not in samples from control mice.

2. Populärvetenskaplig sammanfattning - Popularized summary in Swedish

Som en blixt från klar himmel drabbas en ung, och till synes frisk person av hjärnblödning. Denna stroke leder till demens och efterföljande nya blödningar leder slutligen till en för tidig död. Man har kunnat visa att denna sjukdom beror på att en variant av ett visst protein, cystatin C, tvinnar ihop sig till rep-liknande långa fibriller. Dessa fibriller ansamlas sedan i bland annat blodkärlens väggar, vilket leder till att kärlen brister och det blir en blödning.

Det har visat sig att inte bara cystatin C kan bilda fibriller och orsaka sjukdom, utan även andra proteiner kan förändras på liknande sätt. I denna grupp av sjukdomar återfinns bland annat Alzheimers sjukdom och galna ko-sjukan. Detta innebär att det inte bara är patienterna med cystatin C-relaterad hjärnblödning som skulle kunna få hjälp om man kunde lösa orsaken till sjukdomen, utan detta skulle även kunna leda till framsteg inom forskningen av de andra sjukdomarna.

Men, vad är det då som händer? I denna doktorsavhandling beskriver jag mitt arbete med att ta reda på mekanismen som leder till att fibrillerna bildas i kroppen. För att försöka förstå hur fibrillbildningen sker, har vi tittat på strukturen av proteinet. En monomer av cystatin C liknar en hand som sluter sig om sin tumme. I dimeren av proteinet kan man dock se att den ena handen håller om den andra handens tumme, och *vice versa*. Det vi ser är alltså att en del av proteinet har bytt plats med motsvarande del i en annan molekyl. Denna mekanism brukar kallas ”domain swapping”. I det här fallet har enbart två molekyler bytt delar med varandra, men vad händer om de istället skulle byta delar med ytterligare molekyler? Man skulle kunna tänka sig att vi då kan få en lång kedja som till slut kan bilda en fibrill.

I den här doktorsavhandlingen visar jag att om man ”låser fast” tummen vid sin hand, och därmed stoppar ”domain swapping”, så kan inte dimerer eller fibriller, bildas. Därmed verkar ”domain swapping” vara en viktig mekanism för att cystatin C ska kunna aggregera. Detta har vi

visat genom att på olika sätt stabilisera monomeren. I framtiden skulle man kanske kunna använda samma metod för att ”låsa fast” den flexibla delen av proteinet med ett läkemedel, vilket skulle kunna användas för att bota patienter.

Jag har även undersökt vad som händer innan en fibrill blir till. Vad finns det för mindre aggregat innan de stora fibrillerna bildas? Det visade sig att cystatin C bildar aggregat som ser ut som ringar långt innan fibrillerna bildas. Dessa ringar hade samma utseende som aggregat vilka har hittats vid andra sjukdomar, som till exempel Alzheimers sjukdom. Om man renar upp ringarna kan de bilda fibriller, vilket tyder på att de är byggstenar i fibrillerna. Precis som med dimerer och fibriller hämmas dock bildningen av dessa ringar om ”domain swapping” stoppas, vilket tyder på att denna mekanism är viktig även för bildandet av dessa byggstenar.

Därefter försökte jag att se om dessa ringar kunde hittas hos möss som har samma sjukdom som patienter med cystatin C-relaterad hjärnblödning. Ringar med exakt samma egenskaper som tidigare beskrivits kunde jag inte hitta hos dessa möss. Däremot hittades andra stora aggregat av cystatin C i bland annat blod och hjärna från dessa möss, vilka inte kunde hittas i kontrollmöss. Eventuellt kan även dessa aggregat bildas genom ”domain swapping”, men det får framtiden utvisa.

Denna doktorsavhandling visar alltså på vikten av ”domain swapping” i vissa sjukdomar då fibriller av proteiner ansamlas i kroppen. Genom att känna till denna mekanism får man en möjlighet att i framtiden utveckla läkemedel som specifikt kan hämma ”domain swapping”. Denna strategi skulle kunna utnyttjas i kampen mot ett flertal sjukdomar, såsom till exempel cystatin C-relaterad hjärnblödning och ”galna ko-sjukan”.

3. List of papers

This thesis is based on the following three papers, referred to in the text by their roman numbers.

I. *Prevention of domain swapping inhibits dimerization and amyloid fibril formation of cystatin C. Use of engineered disulfide bridges, antibodies, and carboxymethylpapain to stabilize the monomeric form of cystatin C*. Maria Nilsson, Xin Wang, Sylwia Rodziewicz-Motowidlo, Robert Janowski, Veronica Lindström, Patrik Önnerfjord, Gunilla Westermark, Zbigniew Grzonka, Mariusz Jaskolski, Anders Grubb. **J Biol Chem** 279;24236-24245 (2004)

II. *Fibrillogenic oligomers of human cystatin C are formed by propagated domain swapping*. Maria Wahlbom, Xin Wang, Veronica Lindström, Eric Carlemalm, Mariusz Jaskolski, Anders Grubb. **J Biol Chem** 282;18318-18326 (2007)

III. *Amyloidogenic L68Q cystatin C form high molecular weight species in vivo*. Veronica Lindström, Katarina Håkansson, Anders Grubb, Maria Wahlbom. Manuscript

Paper I and II are reprinted with permission from the copyright owner. In addition to the enclosed papers, I have contributed to the following publication during my PhD studies;

Checking the conformational stability of cystatin C and its L68Q variant by molecular dynamics studies: why is the L68Q variant amyloidogenic? Sylwia Rodziewicz-Motowidlo, Maria Wahlbom, Xin Wang, Justyna Lagiewka, Robert Janowski, Mariusz Jaskolski, Anders Grubb, Zbigniew Grzonka. **J Struct Biol** 154: 68-78 (2006)

4. Abbreviations

Aß	amyloid ß peptide
APP	amyloid precursor protein
ß2m	ß2 –microglobulin
BSE	bovine spongiform encephalopathy
CJD	Creutzfeldt-Jakob disease
DTT	dithiothreitol
Fig.	figure
HCCAA	Hereditary Cystatin C Amyloid Angiopathy
HMWCCIR	high molecular weight cystatin C immunoreactive compound
NMR	nuclear magnetic resonance
PrP	prion protein
PrP^{C}	cellular prion protein
PrP^{Sc}	scrapie-like prion protein
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
vCJD	new variant of Creutzfeldt-Jakob disease
wt	wild type

5. Introduction

In this doctoral dissertation I describe my work to give an insight to the role of the molecular mechanism of domain swapping in amyloidosis. As a short introduction to a vast area, I have chosen to briefly give a few examples of amyloid-related diseases and the proteins involved in the mentioned disorders. Thereafter the molecular mechanism of domain swapping is described. To elucidate the role of this feature in amyloidosis I have performed different experiments using the amyloidogenic protein cystatin C. In this thesis the results from these experiments will be discussed in order to investigate the importance of domain swapping in amyloidosis.

5.1. Amyloidoisis – a protein conformational disease

Amyloidosis is a group of diseases characterized by extracellular deposits of "amyloid" (Ghiso, Jensson *et al*. 1986; Abrahamson, Jonsdottir *et al*. 1992; Westermark, Benson *et al*. 2005). Amyloidosis can be either local or systemic, where the amyloid deposits are localized to a particular organ or found in the whole body, respectively. The term "amyloid" was used by Virchow in 1854 for human deposits of substances with properties similar to cellulose and starch (Virchow 1854; Puchtler and Sweat 1966). He also detected proteins in these deposits, but the protein fraction was not considered to be a part of the amyloid. However, today amyloidosis is characterized as a protein conformational disease, where a change in protein conformation results in self-aggregation and tissue deposition (Carrell and Lomas 1997). The main amyloid component is protein fibrils, which have assumed a non-native ß-sheet-rich configuration, resulting in certain tinctorial and structural properties (Glenner 1980; Westermark, Benson *et al*. 2005). Deposition of usually soluble and physiologically normal proteins and peptides as insoluble fibrils can be formed from at least 25 unrelated proteins, and has consequently been associated with many different human diseases (Wetzel 2002; Dobson 2003; Westermark, Benson *et al*. 2005).

5.1.1. Destabilization of the monomeric protein

The fibrillization reaction can be seen as a nucleation-dependent polymerization pathway with a slow nucleation phase followed by a rapid growth of fibrils, and finally a steady state phase where the fibrils and monomers are in equilibrium (Jarrett, Berger *et al*. 1993; Harper and Lansbury 1997). The duration of the initial lag phase differs from protein to protein and can be influenced in different conditions (Chiti, Webster *et al*. 1999). Seeding a solution with sonicated preformed amyloid fibrils reduces the lag phase for fibril formation significantly, possibly by the elimination of this nucleation step (Harper and Lansbury 1997; Ohnishi and Takano 2004). Seeding has also been suggested to be a mechanism *in vivo* (Johan, Westermark *et al*. 1998; Lundmark, Westermark *et al*. 2002).

To be able to fibrillize, the native monomeric protein first has to be destabilized (Buxbaum 2004). If the native structure of the protein is destabilized, amyloidogenic conformations of the protein may be formed, which fibrillizes when a minimum critical concentration has been exceeded (Harper and Lansbury 1997). The destabilization could be due to destabilizing mutations or increased protein concentration, as a result from increased protein expression or decreased catabolism. For example, individuals with Down syndrome, who carry an extra copy of the genes on chromosome 21, develop amyloidosis of the amyloidogenic protein Aß (See 5.3.) that is encoded on this particular chromosome (Glenner and Wong 1984).

5.1.2. Formation of oligomers

Formation of oligomers has been reported to precede the fibril formation, indicating their possible role as an intermediate in fibrillogenesis. When higher order aggregates are formed, remarkable similarities in appearance have been observed, irrespective of the origin of the precursor protein (Chiti, Webster *et al*. 1999; Lashuel, Hartley *et al*. 2002). Consistent with a common

structure, generated conformation-specific antibodies towards either fibrils (O'Nuallain and Wetzel 2002) or oligomers (Kayed, Head *et al*. 2003) can recognize aggregates obtained from different amyloidogenic proteins.

Oligomers can be seen as ordered aggregates larger than dimers, that have lost the normal function of the protein (Buxbaum 2003). Although soluble oligomers of diverse amyloidogenic proteins have been proposed as productive intermediates in the fibrillogenesis (Lambert, Barlow *et al*. 1998; Bucciantini, Giannoni *et al*. 2002; Lashuel, Petre *et al*. 2002), off-pathway oligomers have also been reported (Baskakov, Legname *et al*. 2002). The importance of the different oligomeric aggregates should not be neglected, since oligomeric precursors of amyloid fibrils may be substantially more toxic than the fibrils themselves (Lambert, Barlow *et al*. 1998; Walsh, Hartley *et al*. 1999; Bucciantini, Giannoni *et al*. 2002; Kirkitadze, Bitan *et al*. 2002; Stefani and Dobson 2003), possibly by permeabilizing membranes (Glabe and Kayed 2006).

5.1.3. Protofibrils and amyloid fibrils

A distinct different morphology from the oligomers can be observed for the protofibrils, that are larger, but still flexible, rod-shaped molecules with a length up to approximately 200 nm and a diameter similar to the mature fibril (Walsh, Lomakin *et al*. 1997). The protofibrils may elongate to form rigid, non-branched mature fibrillar structures with a diameter of 5-13 nm. The fibrils have a common substructure, consisting of 2-6 long and rather rigid protofilaments (2-5 nm in diameter) winding around one another. These protofilaments have been described as continous β-sheets, where their loosely packed peripheral regions may contribute to interactions between the different protofilaments (Jimenez, Guijarro *et al*. 1999). The fibrils also contain β-sheets, which are arranged in a perpendicular fashion relative to the fibre axis (Glenner 1980; Sunde, Serpell *et al*. 1997; Jimenez, Guijarro *et al*. 1999; Chamberlain, MacPhee *et al*. 2000; Jimenez, Nettleton *et al*. 2002).

Amyloid fibrils are known to bind to thioflavine T (LeVine 1993). Fibrils can also bind to the dye Congo red, producing a characteristic red staining and apple-green birefringence when viewed under polarized light (Glenner 1980; Glenner 1980; Westermark, Benson *et al*. 2005).

It is not well known how the accumulation of fibrillar protein deposits affects the human body. The accumulation of protein deposits within a variety of organs and tissues may itself be the major cause of clinical symptoms in amyloidosis (Pepys 2006). The occurrence of hemorrhages in amyloidosis could be due to degeneration of smooth muscle cells in the vessels, which have been associated with the deposition of fibrils (Kawai, Kalaria *et al*. 1993).

5.2. The molecular mechanism of domain swapping

The observation that many different proteins can aggregate and form oligomers and fibrils, with a virtually similar appearance, suggests that the fibrillization event is due to common molecular mechanisms. In contradiction to Anfinsen's hypothesis that one amino acid sequence should only be able to generate one stable protein structure (Anfinsen 1973), both the precursor protein and its fibrils are stable in amyloidosis. However, if the fibrils contain native-like structures it would be possible to have two different stable conformations of the same protein (Guo and Eisenberg 2007). This could be the case if the molecular mechanism of domain swapping is involved in amyloidogenesis, since domain-swapped proteins have the capacity to undergo conformational changes without general unfolding (Bennett, Schlunegger *et al*. 1995; Schlunegger, Bennett *et al*. 1997).

5.2.1. Domain-swapped dimers

The term "domain swapping" was defined in 1994 when crystal structures of monomeric and dimeric diphtheria toxin were compared (Bennett, Choe *et al*. 1994). It was observed that an entire domain of the protein was rearranged upon dimerization, which resulted in an exchange of this domain with the corresponding domain of another molecule. This phenomenon was suggested to be a general mechanism of protein oligomerization. The swapping domain can be either an entire tertiary globular domain or a single α-helix or a ß-strand (Schlunegger, Bennett *et al*. 1997).

The tendency of a protein to undergo domain swapping is related to structural, thermodynamic and kinetic factors (Schlunegger, Bennett *et al*. 1997). A large energy barrier separates the monomeric form from the domain-swapped aggregates, since the protein has to unfold prior to the domain-swapping event. To be able to dimerize, the monomeric protein first must be destabilized, resulting in partly unfolding and formation of an open monomer (Fig. 1A). Thereafter, the open monomer reacts with another open monomer and dimerization occurs (Fig. 1A). This event should be very rare, since it requires the simultaneously formation of two open molecules.

The symmetrical dimer constitutes hence of two domain-swapped monomers. The domain-swapped dimer has the same structure as the original monomer, with the exception of the hinge region (Fig. 1A) (Bennett, Choe *et al*. 1994). The hinge region is the part of the polypeptide chain that links the swapped domain to the rest of its subunits. When the length or the flexibility of the hinge region changes, *e.g.* prolines are replaced by alanines, the capacity to undergo domain swapping is influenced (Schymkowitz, Rousseau *et al*. 2000; Rousseau, Schymkowitz *et al*. 2003). It has been suggested that domain-swapped aggregates may interact within the hinge region in domain swapped fibrils (Janowski, Kozak *et al*. 2005; Sambashivan, Liu *et al*. 2005).

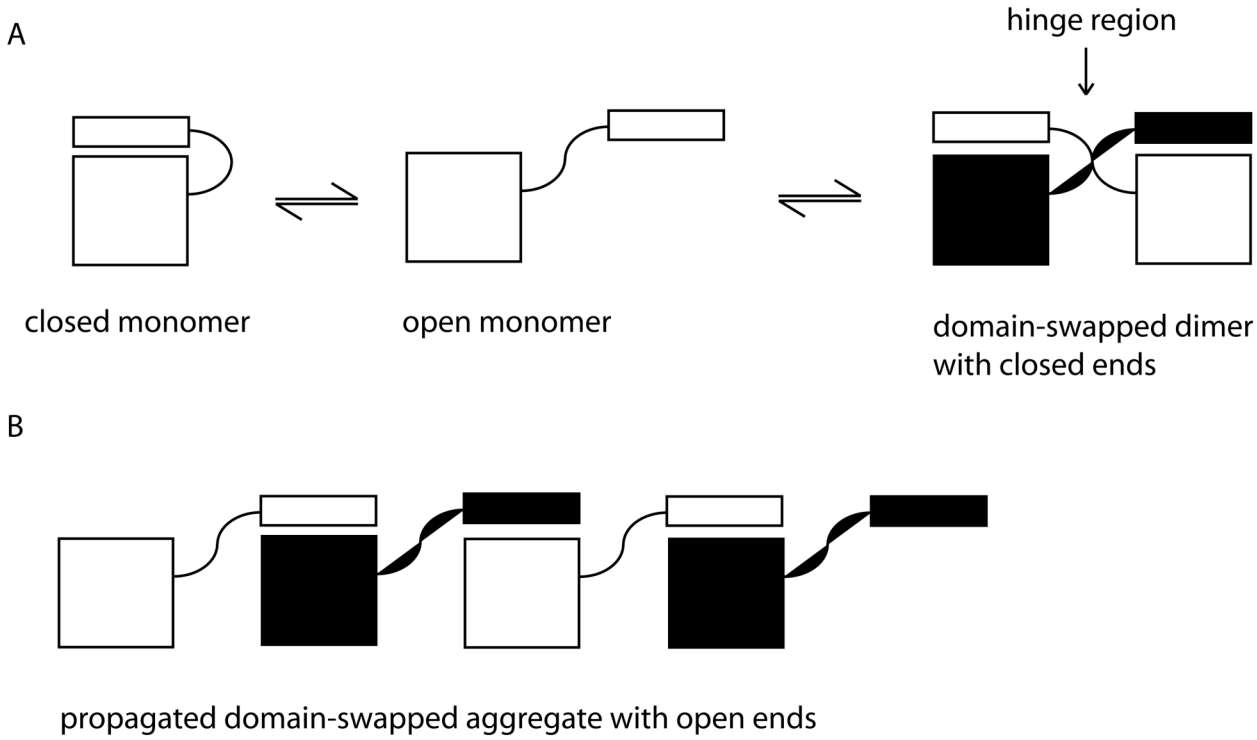

Figure 1. The process of domain swapping. (A) When a protein undergoes domain swapping, the monomeric protein first has to unfold in order to produce an "open monomer", which thereafter can dimerize. (B) When each molecule interacts with two neighbouring molecules, propagated domain swapping occurs.

5.2.2. Propagated domain swapping

Larger oligomers can be formed by domain swapping when each molecule interacts with two neighbouring molecules by providing a domain to one molecule and accepting the corresponding domain from another molecule (Fig. 1B) (Bennett, Choe *et al*. 1994). This feature will, in this thesis, be denoted "propagated domain swapping", although other terms have been used in the literature, such as "runaway domain swapping" (Guo and Eisenberg 2006).

Propagated domain swapping may occur when an aggregate is formed in such way that it is not closed-ended (Fig. 1A), but open-ended (Fig. 1B) (Bennett, Choe *et al*. 1994; Jaskolski 2001). The progression of oligomerization can be an autocatalytic process, where the growing polymer always ends with an "open" molecule, which will bind to an open monomer as soon as possible (Schlunegger, Bennett *et al*. 1997). Hence, once the formation of the domain-swapped polymer has

started, its growth should be rapid. This is in agreement with the known growth rate of fibrils with a long lag phase followed by an exponential growth of the fibrils (See 5.1.1.).

5.3. Alzheimer disease

In 1907 the German physician Alois Alzheimer described changes in the brain tissue of a woman who had died of mental illness (Alzheimer 1907). Later this neurodegenerative disorder was denoted Alzheimer disease. The disease is associated with unusual behavior, personality changes, and a decline in thinking abilities. Alzheimer disease is the most common form of dementia in adults. In 2003 approximately 28 million persons were estimated to suffer from Alzheimer disease worldwide (Wimo, Jonsson *et al*. 2006). It has been characterized by extensive neuronal loss and synaptic changes in areas of the brain essential for cognitive and memory functions (Parihar and Hemnani 2004). Alzheimer disease appears in both sporadic and autosomal dominant forms, and has been associated with Down syndrome (Glenner and Wong 1984), sporadic cerebral amyloid angiopathy and normal aging (Maruyama, Ikeda *et al*. 1990).

5.3.1. The amyloid ß peptide

It has been shown that brains from Alzheimer disease patients contain extracellular deposits of the small (4.3 kDa) hydrophobic amyloid ß peptide (Aß) (Glenner and Wong 1984). This peptide is derived from the amyloid precursor protein (APP), which can either be secreted in a soluble form or cleaved to Aß in different lengths, *e.g.* $Aß_{1-40}$, $Aß_{1-42}$. The Aß is known to be a normal constituent of body fluids, such as blood plasma and CSF of healthy persons (Seubert, Vigo-Pelfrey *et al*. 1992), but can also be highly amyloidogenic and form aggregates. The $Aß_{1-42}$ is known to be more amyloidogenic than $Aß_{1-40}$ (Jarrett, Berger *et al*. 1993). It has been suggested that the

amyloidogenic conformation of Aß consists of mainly ß-sheets, while the more stable conformation consists of both α-helical and ß-sheet structures (Soto, Castano *et al*. 1995).

The concentration of Aß in CNS is regulated by different mechanisms. It has been suggested that the protein can be eliminated by both drainage with the interstititial fluids (Weller, Massey *et al*. 1998) and by active transport across the blood-brain barrier by different proteins (Shibata, Yamada *et al*. 2000). An age-dependent reduction of the clearance would lead to elevated Aß levels in the brain, favoring amyloidosis.

5.3.2. Other proteins involved in Alzheimer disease

It has been suggested that the aggregation of Aß induces other disease-related events in the brain, *e.g.* hyperphosphorylation of the tau protein in the intracellular neurofibrillary tangels (Geula, Wu *et al*. 1998). It has also been observed that the risk of developing Alzheimer disease can be associated with the presence of certain isoforms of the lipoprotein apoE (Corder, Saunders *et al*. 1993), possibly by their function as chaperon-like proteins (Wisniewski and Frangione 1992).

Immunohistochemical studies have shown colocalization of the cysteine protease inhibitor cystatin C with Aß in the cerebral vasculature of patients with Alzheimer disease (Levy, Sastre *et al*. 2001), cerebral amyloid angiopathy (Maruyama, Ikeda *et al*. 1990), and progressive sporadic inclusion body myositis (Vattemi 2003). It has been suggested that cystatin C might have a protective role, since cystatin C has been observed to decrease the level of oligomerization and fibrillization of Aß *in vitro* (Sastre, Calero *et al*. 2004; Selenica, Wang *et al*. 2007) and *in vivo* (Kaeser, Herzig *et al*. 2007). It has also been suggested that an elevated cystatin C expression in Alzheimer disease may have a neuroprotective effect of neural cells to oxidative stress (Nishiyama, Konishi *et al*. 2005). The oxidative stress induces leakage of lysosomal cysteine proteases into the

cytosol, which in its turn leads to cell death. However, the activity of these proteases can be inhibited by the cysteine protease inhibitor cystatin C (See 5.6.2.).

5.4. Prionoses

The prionoses are a group of diseases, including Creutzfeldt-Jakob disease (CJD) in humans, bovine spongiform encephalopathy (BSE) in cattle and scrapie in sheep (Prusiner, Scott *et al*. 1998; Soto 1999; Johnson 2005). Prion disorders are characterized by dementia, neuron loss, and spongiform degeneration of the brain, and in some cases by amyloid plaques. CJD may be sporadic (85% of the cases), familial (10%) or iatrogenic (5%), and usually the patients are between 50 and 75 years old (Brown, Gibbs *et al*. 1994; Soto and Saborio 2001). However, in 1996 a new variant of CJD (vCJD) was observed, affecting young persons (average 29 years old) and with a longer duration of illness (14 months instead of 4 months in sporadic CJD) (Will, Ironside *et al*. 1996; Hill, Desbruslais *et al*. 1997). This variant of CJD has been associated with industrial cannibalism, where humans have eaten beef from cattles, which have been fed with meat from other cattles (Bruce, Will *et al*. 1997).

5.4.1. The prion protein

In 1982 the term "prion" was proposed to denote a small, proteinaceous and infectious particle involved in prionoses (Prusiner 1982). This prion protein (PrP) has been observed to accumulate in the brain of affected patients (Prusiner, McKinley *et al*. 1983). The cellular PrP (PrP^{C}) may be either in its normal monomeric α-helical state or bound to a chaperon-like protein, named protein X (Cohen, 1994). The complex between PrP^{C} and protein X can undergo a conformational change which results in the formation of the pathogenic form of PrP (PrP^{Sc}, where Sc is derived from scrapie) (Kaneko, Zulianello *et al*. 1997). PrP^{C} is thought to have an α-helix-rich

conformation, while PrP^{Sc} is a ß-sheet-rich conformation, indicating that the conversion from PrP^{C} to PrP^{Sc} involves a major conformational transition (Pan, Baldwin *et al*. 1993). Thereafter PrP^{Sc} may aggregate into insoluble multimers.

It has been shown that inoculation of tissues from animals, suffering from prion disease, causes disease in the recipient host (Hill, Desbruslais *et al*. 1997). It has been hypothesized that an infecting inoculum containing PrP^{Sc} may interact with the PrP^{C} of a host and thereby catalyzing the conversion of PrP^{C} to PrP^{Sc}. Indeed, it has been observed a relationship between the amount of ß-sheet-structures of PrP and infectivity *in vitro* (Safar, Roller *et al*. 1993) and *in vivo* (Tremblay, Ball *et al*. 2004).

5.4.2. Domain swapping of the prion protein

According to the NMR analysis, monomeric human prion protein consists of three α-helices (Fig. 2A) (Zahn, Liu *et al*. 2000; Calzolai and Zahn 2003). However, when the monomeric human prion protein was crystallized, the structure demonstrated a covalently dimerized protein (Knaus, Morillas *et al*. 2001). Although a starting material of monomeric protein was used, domain-swapped dimers were formed during the process of crystallization (Fig. 2B). It was observed that the swapping domain constitutes of an α-helix and that the hinge region in the dimeric protein form a small antiparallel ß-sheet. Many of the known point mutations involved in familial prionoses are located in this swapping part of the protein, which might affect the equilibrium between the monomer and the dimer. Also the binding between the prion protein and protein X has been suggested to be in the swapping area (Kaneko, Zulianello *et al*. 1997).

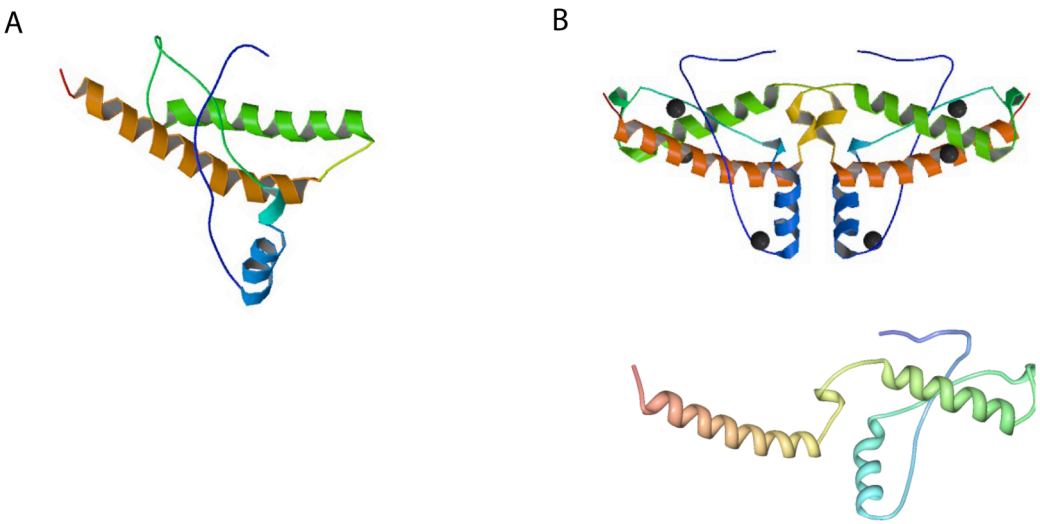

Figure 2. The structure of the human prion protein. (A) The structure of monomeric human prion protein deduced from NMR analysis (Calzolai and Zahn 2003). (B) The crystal structure of dimeric human prion protein and its "open" monomer (Knaus, Morillas *et al*. 2001). The figure was generated from coordinates deposited in the Protein Data bank (1hjm and 1i4m, respectively) and the MBT Protein Workshop application available from the Research Collaboratory for Structural Bioinformatics (RCSB) (Moreland, Gramada *et al*. 2005).

Although the mechanism of domain swapping may be involved in the fibrillogenesis of prions, it is not known how the smaller subunits of the fibrils are organized. The observation of the domain-swapped dimers in the crystallized form of the protein suggests a dramatic conformational change since one disulfide bond within the protein must be reduced prior to formation of the open monomer. After the event of domain swapping, two disulfide bonds must be reformed within the newly formed dimer. In contrast to the proposed importance of prion dimers in the fibrillogenesis, prion fibrils have been demonstrated to grow rapidly by addition of monomers to fibril ends (Collins, Douglass *et al*. 2004). In addition, it has been hypothesized that a domain-swapped prion trimer is involved in the fibrillization (Yang, Levine *et al*. 2005). In this case, domain swapping will stabilize the trimer of PrP^{Sc} by reducing constraints in proline containing loops and by increasing intermonomer hydrogen bonding.

5.5. ß2-microglobulin amyloidosis

Over one million people worldwide who are receiving heamodialysis are at risk of developing symptomatic ß2-microglobulin (ß2m) amyloidosis, since it is a frequent complication of long-term heamodialysis (Hirschfield 2004). In heamodialyzed patients, plasma concentrations of ß2m may rise up to 60 times above the normal level (Vincent, Revillard *et al*. 1978), since its clearance relies on renal catabolism and excretion. Therefore, during long-term heamodialysis, this protein might accumulate as amyloid fibrils in the bones and joints, causing a variety of arthropathies and pathological fractures (Gejyo, Yamada *et al*. 1985). However, since ß2m fibrillizes easily *in vitro* in the presence of Cu^{2+}, it has been suggested that not only the protein concentration, but also the level of copper, is of importance in the fibrillogenesis of ß2m (Morgan, Gelfand *et al*. 2001).

5.5.1. The structure of ß2-microglobulin

The amyloidogenic protein ß2m is part of the major histocompatibility class I complex found on the surface of all nucleated cells. In addition, this protein circulates in blood in its monomeric form, containing 99 residues and with a molecular weight of 11.8 kDa (Gejyo, Yamada *et al*. 1985). The crystal structure of ß2m consists of two antiparallell ß-sheets, one with four ß-strands and one with three ß-strands, connected with a disulfide bond (Björkman, Saper *et al*. 1987; Trinh, Smith *et al*. 2002). According to NMR studies, fibril-promoting conditions induce a partially unfolding of ß2m where its N- and C-terminus are destabilized, implying the importance of the presence of the disulfide bond for the stability (McParland, Kalverda *et al*. 2002). In addition, it has been shown that ß2m fibrillizes easily in oxidizing *in vitro* conditions, while an increased flexibility of the reduced molecule results in the production of dead-end products (Katou, Kanno *et al*. 2002).

5.5.2. Domain swapping of ß2-microglobulin

According to NMR analysis of ß2m fibrils, the core of the fibrils are as rigid as the core of native globular ß2m, implying that a major part of the native fold is preserved in the fibrils (Hoshino, Katou *et al*. 2002). It has been suggested that fibrillization of ß2m occurs when domain-swapped native-like precursors assemble (Eakin, Attenello *et al*. 2004). The fibrillization of ß2m initiates when the native protein turns to an alternative conformation, which is catalyzed by Cu^{2+} (Eakin, Attenello *et al*. 2004). The change in conformation results in an immediate assembly of the molecules to different domain-swapped oligomers (Eakin, Attenello *et al*. 2004; Eakin, Berman *et al*. 2006). In accordance to these experimental data, molecular dynamic analysis has shown that the oxidized ß2m forms a domain-swapped structure, in contrast to the reduced form of ß2m (Chen and Dokholyan 2005). When the domain swapped aggregate is produced, the former intra-molecular interactions are replaced by stabilizing inter-molecular interactions between the monomers. This will also be the case if propagated domain swapping occurs between several monomers of ß2m.

5.6. Cystatin C amyloidosis

The Icelandic physician Arni Arnason described in 1935 patients suffering from fatal cerebral hemorrhage in early adulthood (Arnason 1935; Olafsson and Grubb 2000; Palsdottir, Snorradottir *et al*. 2006). The disease "Hereditary cystatin C amyloid angiopathy" (HCCAA) has been characterized as an autosomal dominant hereditary disease, where all patients are from a number of families living in the same region of Iceland (Jensson, Gudmundsson *et al*. 1987). Usually the first hemorrhage occurs before the age of 30 years. The presenting symptoms are acute onset of headache, nausea, focal neurological signs, and loss of consciousness, which occurs in normotensive and previously healthy persons. Patients, who survive the first hemorrhage, suffer

subsequently from more hemorrhages, dementia and finally death before the age of 50 (Jensson, Gudmundsson *et al*. 1987).

In patients suffering from HCCAA extensive amyloid deposition of cystatin C has been detected in small cortical arteries and arterioles, The deposits have been found mainly in the vessel walls, where the smooth muscle cells are destroyed, implying that the hemorrhage may be due to weakening of the arterial walls (Wang, Jensson *et al*. 1997). Cystatin C deposits have also been found in other tissues, such as the spleen (Löfberg, Grubb *et al*. 1987).

5.6.1. L68Q cystatin C

HCCAA is characterized by abnormally low levels of cystatin C in the CSF (Grubb, Jensson *et al*. 1984). The main component of the amyloid has been shown to be fibrils of L68Q cystatin C, where a single mutation causes the substitution of leucine to glutamine in cystatin C (Ghiso, Jensson *et al*. 1986). The fact that the mutation of an A to a G abolishes an *Alu*I restriction enzyme site can be used to diagnose HCCAA patients (Palsdottir, Abrahamson *et al*. 1988; Abrahamson, Olafsson *et al*. 1990). The altered residue in L68Q cystatin C is located in a hydrophobic pocket of the protein, where the introduction of a longer and hydrophilic glutamine side chain will cause a destabilization of the monomer, leading to structural rearrangements and an increased ability to dimerize (Janowski, Kozak *et al*. 2001). Indeed, this agrees with the observations that L68Q cystatin C forms dimers more easily than wt cystatin C *in vitro* (Abrahamson and Grubb 1994) and in human body fluids (Bjarnadottir, Nilsson *et al*. 2001). In fact, significant amounts of extracellular dimers are present only in pathological conditions. According to molecular dynamic analysis, the stability of the L68Q cystatin C dimer may be related to salt bridges present in the dimer but not in the monomer (Rodziewicz-Motowidlo, Wahlbom *et al*. 2006).

5.6.2. The cysteine protease inhibitor cystatin C

Cystatin C is a cysteine protease inhibitor with 120 amino acids (Grubb and Löfberg 1982). It contains two disulfide bonds close to the C-terminal of the protein (Barrett, Davies *et al*. 1984). Cystatins in general are known to fold as a long α-helix running across a large five-stranded anti-parallell β-sheet (Fig. 3A) (Bode, Engh *et al*. 1988). Cystatin C has been found in all human tissues and body fluids examined (Grubb 2000), where it acts as a potent, reversible inhibitor of cysteine proteases belonging to the papain (C1) (Abrahamson, Barrett *et al*. 1986; Hall, Håkansson *et al*. 1995) and the legumain (C13) families (Alvarez-Fernandez, Barrett *et al.* 1999). The papain-binding site of cystatin C includes the N-terminal, a central loop (L1) and a second C-terminal loop (L2) (Fig. 3A). However, legumain binds to the opposite side of the cystatin C molecule, where at least the loop connecting the α-helix with the ß2-strand is involved in the inhibition (Fig. 3A) (Alvarez-Fernandez, Barrett *et al*. 1999).

5.6.3. Domain swapping of cystatin C

Monomeric human cystatin C produce cystatin C dimers upon crystallization (Janowski, Kozak *et al*. 2001; Janowski, Abrahamson *et al*. 2004; Janowski, Kozak *et al*. 2005). Just as in the case of crystallization trials of monomeric prions (See 5.4.2.), the starting material of monomeric cystatin C (Fig. 3A) formed domain-swapped dimers during the process of crystallization (Fig. 3B). According to NMR analysis, the dimeric fold of L68Q cystatin C is virtually identical to the dimer of wt cystatin C (Gerhartz, Ekiel *et al*. 1998), suggesting that also the dimer of L68Q cystatin C is formed by domain swapping. The domain that undergoes swapping consists of the α-helix and its two flanking β-strands (β1 and β2) (Fig. 3B). It is connected to the C-terminal part of the protein (strands β3-β5) by a hinge region that in the monomeric molecule forms the first β-hairpin loop (L1), which is involved in the inhibition of papain. Consequently, when cystatin C undergoes

domain swapping, the papain-binding site is abolished, while the legumain-binding site is preserved (Alvarez-Fernandez, Barrett *et al*. 1999; Janowski, Kozak *et al*. 2001).

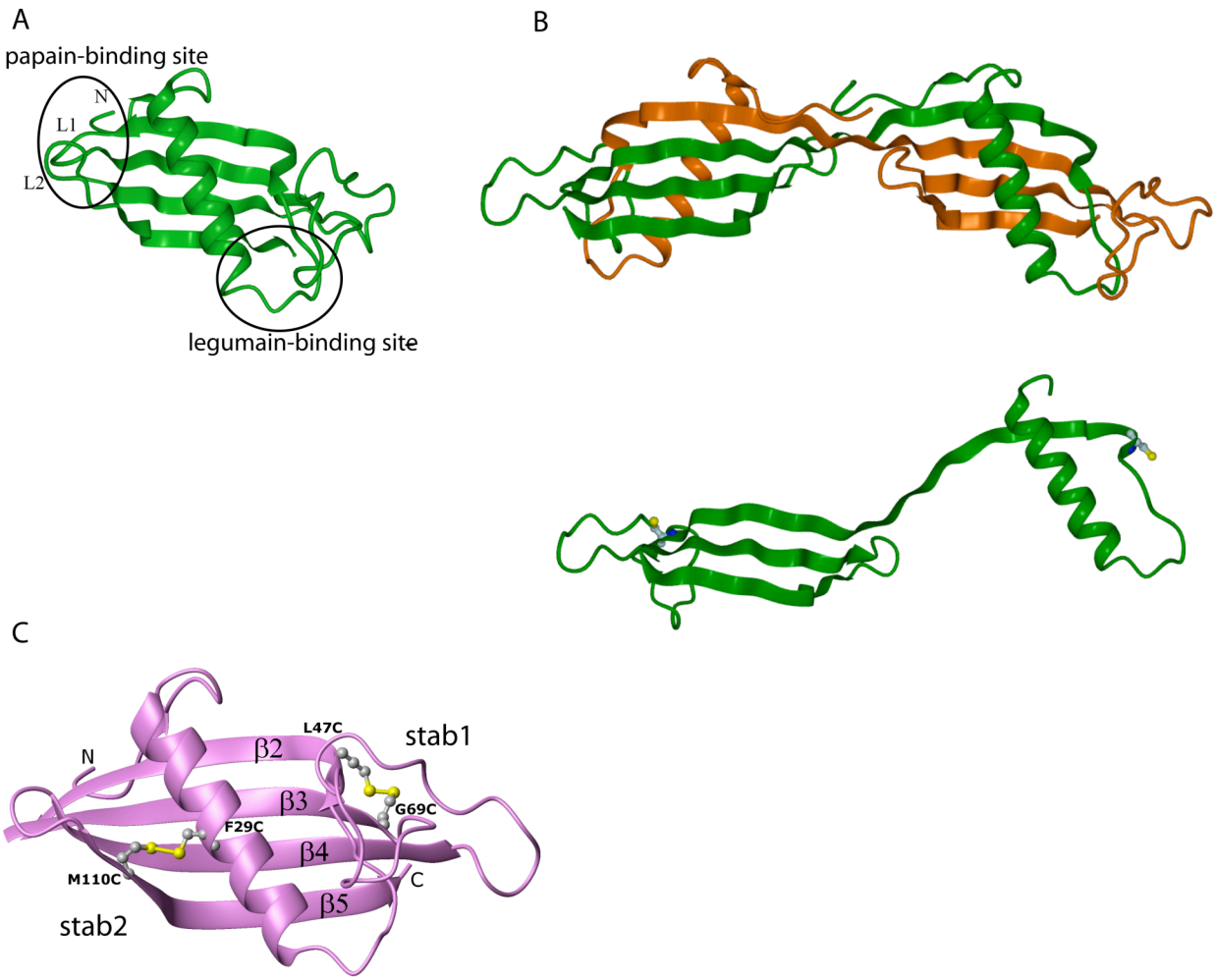

Figure 3. The structure of human cystatin C. (A) The structure of monomeric human cystatin C deduced from the known structures of chicken cystatin (Bode, Engh *et al*. 1988) and dimeric wt cystatin C (Janowski, Kozak *et al*. 2001; Janowski, Kozak *et al*. 2005). (B) The crystal structure of domain-swapped dimeric human cystatin C and its "open" monomer (Janowski, Kozak *et al*. 2001). (C) A model of the stabilized monomer, where disulfide bonds have been introduced in order to prevent domain swapping. The mutations F29C/M110C were designed to introduce a connection between the α-helix and the β2-strand, while the mutations L47C/G69C were designed to introduce a disulfide bond between the β2- and the β3-strands. The figures have been generated from coordinates deposited in the Protein Data Bank (1G96, 1tij and 1cew, respectively), using the program MOLMOL (Koradi, Billeter *et al*. 1996), and have been published in Paper I and II.

Propagated domain swapping has been proposed to be involved in the fibrillization of cystatin C (Janowski, Kozak *et al*. 2001; Jaskolski 2001). In addition, different domain-swapped oligomers may play a role in the fibrillogenesis. The crystal structure of N-truncated wt cystatin C shows two tetramers stacked on top of each other (Janowski, Abrahamson *et al*. 2004). The tetramers consist of two dimers interacting by their hinge region, which has also been observed in the crystal structure of full-length wt cystatin C (Janowski, Kozak *et al*. 2005). Although the observed dimers were all symmetrical within each dimer molecule, the hinge regions had slightly different conformations resulting in different dimer conformations (Janowski, Abrahamson *et al*. 2004). However, anti-parallell ß-interactions were always present between the hinge region and the ß2 and ß3 strands, irrespective of the conformation of the dimeric fold (Janowski, Kozak *et al*. 2005).

It has also been suggested that the tetramers may assemble into infinite structures with all ß-chains in perpendicular orientation to the axis of propagation (Janowski, Kozak *et al*. 2005). This could be of importance in the fibrillogenic context, since cross-ß-structure is believed to constitute the core of the fibril (See 5.1.3.). However, it is not evident how this observation would be compatible with the occurrence of propagated domain swapping. Tetrameric intermediates have indeed been confirmed to be present prior to *in vitro* fibril formation of chicken cystatin (Sanders, Jeremy Craven *et al*. 2004).

5.7. Treatment of amyloidosis

Today no medicins to cure amyloidosis is available. In the case of Alzheimer disease, pharmacotherapy consists of different compounds with the aim to reduce the symptoms (Lleo, Greenberg *et al*. 2006). Acetylcholinesterase inhibitors, such as donepezil and rivastigmine, reduce degradation of the neurotransmitter acetylcholine, which is known to exist in a low concentration in

patients suffering from Alzheimer disease. These substances produce a small symptomatic improvement, but do not have any effect on the disease progression. So is the case also for antiglutaminergic substances, such as memantine, which block glutaminergic neurotransmission.

6. The present investigation

The aim of my PhD project has been to investigate the molecular mechanism of domain swapping in amyloidosis. This will not just give an insight into this mechanism, but also enhance our understanding of the molecular pathophysiology of amyloid fibril formation and its prevention. The investigation was performed using the amyloidogenic protein cystatin C. HCCAA can be used as a model for studying amyloidosis in general, since one single point mutation in one certain gene causes amyloidosis (Ghiso, Jensson *et al*. 1986; Palsdottir, Abrahamson *et al*. 1988; Palsdottir, Abrahamson *et al*. 1989). To study the mechanism of domain swapping, cystatin C is also a good choice since both its monomer and domain-swapped dimer can be isolated and studied in solution (Abrahamson and Grubb 1994). Therefore, mutated variants of cystatin C with a decreased capacity to domain swap were established and their propensities to dimerize, oligomerize and fibrillize were determined. In addition, investigations were performed in order to detect and characterize possible oligomers *in vivo* and *in vitro*. Thereafter, the possible role of domain swapping in the formation of oligomers was investigated.

6.1. Establishment of stabilized cystatin C mutants (Paper I)

Considering the proposed model of propagated domain swapping (Fig. 1B), it seems possible that inhibiting this process would not only suppress dimerization, but also oligomerization and fibrillization of cystatin C. To test this hypothesis, cystatin C mutants with a decreased flexibility of the swapping domains were established. Appropriate point mutations (L47C/G69C

and F29C/M110C, respectively) were introduced by site directed mutagenesis into the vectors encoding wt cystatin C and L68Q cystatin C, respectively. Hence, an intrinsic stabilizing disulfide bond between either the strands β2 and β3 (named stab1) or the α-helix and the β5-strand (named stab2) was introduced into wt cystatin C and L68Q cystatin C, respectively (Fig. 3C).

It was possible to express and purify four different variants of cystatin C, two potentially stabilized variants of wt cystatin C and two of L68Q cystatin C. Physicochemical characterization, including investigations of protease inhibition, of the stabilized cystatin C mutants gave strong support for the variants produced being those intended and, in addition, correctly folded in spite of the fact that new disulfide bonds have been introduced into the molecules. In the case of wt cystatin C stab1, this has been further confirmed when the crystal structure of the protein has been determined (unpublished data).

6.2. Domain swapping is a prerequisite of dimerization (Paper I)

The cystatin C dimer is the only molecular form of cystatin C that has been detected in body fluids besides monomeric cystatin C and amyloid fibrils (Bjarnadottir, Nilsson *et al*. 2001). Dimeric wt cystatin C has also been detected intracellularly in Chinese hamster ovary cells, suggesting that aggregates of cystatin C remain stable in the endoplasmic reticulum during intracellular transports (Merz, Benedikz *et al*. 1997). Although dimeric cystatin C could be an intermediate in the transformation of monomeric cystatin C to amyloid fibrils, an alternative hypothesis is that the dimers are potential dead-end products on the fibrillization pathway (Janowski and Jaskolski, 2001).

L68Q cystatin C has an increased propensity to form dimers (Abrahamson, 1994), but also wt cystatin C has been shown to dimerize *in vitro* and the rate of the dimerization can be increased by raising the temperature, lowering the pH, or using conditions of mild chemical

denaturation (Ekiel and Abrahamson 1996). When subjecting the different stabilized cystatin C variants to conditions promoting dimerization of wt cystatin C, no dimerization was observed. In the case of L68Q cystatin C and its stabilized variants, the results were in concordance with the one of wt cystatin C. Although a significant amount of L68Q cystatin C dimers could be detected, the two stabilized variants of L68Q cystatin C did not produce any dimers at all.

The results of the dimerization experiments support thus the idea that increasing the intrinsic stability of cystatin C by a disulfide bridge between the strands β2 and β3 or between the β5-strand and the α-helix will increase the stability of the proteins. This limited flexibility will inhibit dimer formation of both wt and L68Q cystatin C. This is in accordance with previous results, suggesting that dimers of wt cystatin C are formed by domain swapping and that the flexibility of the α-helix and the β2 strand is important in this event (Janowski, Kozak *et al*. 2001; Janowski, Abrahamson *et al*. 2004; Janowski, Kozak *et al*. 2005). It also argues in favour of the presence of structural similarities between the dimers of L68Q and wt cystatin C, which has been suggested from NMR analysis (Gerhartz, Ekiel *et al*. 1998).

6.3. In vitro oligomerization of cystatin C (Paper II)

It is a matter of debate as to whether or not it is the fibrils or smaller oligomers that contribute to the pathogenic symptoms of amyloid diseases (Lansbury 1999; Ellis and Pinheiro 2002). It has been observed that when different proteins fibrillize *in vitro*, structures that form early in the aggregation process are toxic to cells, whereas the fibrils themselves are non-toxic (Ellis and Pinheiro 2002). In addition, off-pathway oligomers of Aβ have also been demonstrated to exhibit a cytotoxic effect (Lambert, Barlow *et al*. 1998). To improve the knowledge of the molecular mechanism of the production of amyloid fibrils, determination of the structures of possible

intermediates in the transformation process is hence necessary. How is one intermediate transformed into the next? What is the role of on-pathway and off-pathway oligomers?

Therefore, we wanted to investigate whether or not *in vitro* oligomers of cystatin C can be formed. Monitoring different incubation solutions, containing cystatin C, before the formation of amyloid fibrils revealed the presence of oligomers at some incubation conditions. According to electron microscopy, the majority of the wt cystatin C oligomers were symmetrical doughnut-shaped objects with a central hole. The outer diameter was approximately 13.4 nm, the width of the ring 5.4 nm, and the inner diameter about 2.7 nm. The L68Q cystatin C oligomers had virtually the same form and dimensions. The appearance of these doughnut-shaped oligomers resembles that described for oligomers of other amyloidogenic proteins, such as Aβ (Lashuel, Hartley *et al*. 2002; Lashuel, Hartley *et al*. 2003). These results support the notion that, like the fibrils (Chiti, Webster *et al*. 1999), also the oligomers share a similar highly organized multimolecular architecture, regardless of the structure of the native protein. Consistent with the idea of a common oligomeric structure, Kayed *et al.* have found that oligomers of several unrelated proteins all bound to polyclonal antibodies raised against Aß oligomers, whereas those antibodies did not bind to the native monomeric proteins (Kayed, Head *et al*. 2003). Indeed, these oligomer-specific antibodies also bound to the oligomers of wt cystatin C, but not to monomeric or dimeric cystatin C.

6.4. Domain swapping is a prerequisite of oligomerization (Paper II)

To investigate the role of domain swapping in the oligomerization process, the capacity of the stabilized wt cystatin C variants (See 6.1.) to oligomerize was tested. Incubation of the stabilized variants of monomeric wt cystatin C, at conditions producing large amounts of oligomers from wt cystatin C, did not result in any detectable production of oligomers. Prevention of domain swapping, therefore, seems to suppress the formation of oligomers.

However, we also wanted to test whether or not propagated domain swapping was involved in the oligomerization process. Therefore, redox experiments were performed using stabilized wt cystatin C variants. The intrinsic stability of the stabilized cystatin C monomers is due to an inserted extra disulfide bond. Reduction of this disulfide bond will therefore decrease the stability of the monomeric protein, and it should be as prone to oligomerization as non-stabilized wt cystatin C. Indeed, when the stabilized cystatin C variants were incubated in the presence of the reducing agent DTT at conditions producing oligomers from wt cystatin C, oligomers could be observed. The apparent size of the oligomers was virtually similar to that observed for oligomers of wt cystatin C.

The oligomers formed from stabilized wt cystatin C were observed to be more stable in SDS than the one formed from wt cystatin C. However, reduction of the stabilized oligomers resulted in their complete dissociation into monomers. Since no dimers were detected in this experiment, it is not likely that the oligomers contain interacting dimers. The results are hence compatible with the formation of a chain of cystatin C molecules linked by propagated domain swapping (Fig. 1B) and stabilized by disulfide bonds between the swapped domains of adjacent protein molecules.

6.5. Domain swapping is a prerequisite of fibrillization (Paper I and II)

To investigate the fibrillization capacity of cystatin C, *in vitro* systems promoting fibril formation of the protein were established using buffers of pH 2.0 and 4.0. Considering that possibly any protein is capable to fibrillize, given that certain criteria are met, one could suggest that all proteins investigated may fibrillize in the *in vitro* system used, but the propensity to do so under given circumstances varies between different proteins (Dobson 1999). Fibrillization of wt cystatin C was observed upon incubation of monomeric cystatin C. Incubation at pH 4.0 of monomeric wt cystatin C, at a concentration of 3 mg/ml, produced amyloid fibrils after about 3 weeks, while incubation of L68Q cystatin C, at a concentration of 0.6 mg/ml, resulted in the production of

amyloid fibrils within only three days. This result strongly suggests that the substitution of glutamine for leucine in L68Q cystatin C not only results in an enhanced dimerization rate of the molecule *in vitro* (Abrahamson and Grubb 1994), but also in an increased propensity to form amyloid fibrils.

To test the importance of domain swapping in the fibrillization event, the fibrillization capacity of the stabilized variants was investigated. Stabilized wt cystatin C variants produced fibril amounts corresponding to less than 20% of the amount produced by wt cystatin C at pH 2.0, while no fibrils at all could be observed when the stabilized variants were incubated at pH 4.0. The same fibril inhibiting effect was achieved whether the strands β2 and β3 or the β5-strand and the α-helix were stabilized. Hence, domain swapping is a prerequisite of fibrillization of cystatin C. However, we were not able to investigate whether the fibrils are formed by association between domain-swapped aggregates, such as dimers or oligomers, or by propagated domain swapping.

6.6. Fibrillogenic oligomers (Paper II)

Oligomers are believed to represent intermediates in the pathway of fibril formation (See 5.1.2.). It has been shown in inhibiting assays, that some inhibitors target specifically either oligomers or fibrils, whereas other inhibitors inhibit both aggregation steps, implying that oligomers may be either on-pathway or off-pathway (Necula, Kayed *et al*. 2007). Hence, the *in vitro* oligomers produced from cystatin C (See 6.3.) might be either fibril-assembly intermediates or off-pathway products. We, therefore, tested whether incubation of purified oligomers would result in fibril formation. Indeed, the fibrillization capacity of the purified wt cystatin C oligomers was observed to be much higher than that of monomeric cystatin C, strongly suggesting that the oligomers are fibril-assembly intermediates and not off-pathway products.

The mechanism through which the oligomers form amyloid fibrils is unknown. One possibility is that the doughnut-shaped oligomers attach on top of each other to form "tubular" protofibrils or mature fibrils. This would be in agreement with observations suggesting a hollow core of amyloid fibrils (Serpell, Sunde *et al*. 1995; Jimenez, Guijarro *et al*. 1999). In this model, propagated domain swapping would contribute to the formation of each oligomeric subunit, but not necessarily to the stacking of the subunits along the fibril axis. Interactions between the different subunits might be similar to those described in the crystal structure of wt cystatin C, where the ß2 and ß3 strands together with the hinge region may interact with other domain-swapped molecules (See 5.6.3.) (Janowski, Abrahamson *et al*. 2004).

Another possibility is that the oligomer rings open up and wind around each other, forming short protofibrils with the same width as the mature fibril. The short rod-like protofibrils would be open-ended (Fig. 1B) and recognize each other with high affinity, contributing thereby to the elongation step and resulting in mature fibrils. Propagated domain swapping would, in this scenario, be a mechanism for the attachment of adjacent monomers to each other not only within the oligomers but also along the protofibrils and mature fibrils.

6.7. In vivo oligomerization (Paper III)

To improve the knowledge of *in vivo* amyloidosis, animal model systems can be used (Håkansson 1998; Burgermeister, Calhoun *et al*. 2000; Pawlik, Sastre *et al*. 2004; Håkansson 2007; Kaeser, Herzig *et al*. 2007). A mouse model to investigate cystatin C amyloidosis might be suitable, since both human and mouse cystatin C are expressed in all nucleated cells, resulting in a widespread and general tissue distribution of the protein (Löfberg and Grubb 1979; Abrahamson, Olafsson *et al*. 1990; Huh, Nagle *et al*. 1995). In addition, the enzyme inhibitory properties of human and mouse cystatin C are similar (Håkansson, Huh *et al*. 1996). However, no transgenic

mouse strain expressing human L68Q cystatin C have so far been shown to produce amyloid deposits of cystatin C (Pawlik, Sastre *et al*. 2004; Håkansson 2007; Kaeser, Herzig *et al*. 2007).

Nevertheless, we wanted to test whether high molecular weight cystatin C immunoreactive components can be demonstrated *in vivo*. If such aggregates could be found, it would be of interest to compare them with the *in vitro* cystatin C oligomers produced (See 6.3.). Therefore, we analyzed samples from transgenic mice, which are known to express human L68Q cystatin C, as well as mouse cystatin C (Håkansson 1998; Håkansson 2007). Indeed, one large cystatin C immunoreactive band appeared upon immunoblotting of agarose gel electropherograms of brain homogenates from transgenic, but not from wt mice. According to gel chromatography analysis, this band corresponds to a high molecular weight species with a mass between 100 and 150 kDa. No bands corresponding to this high molecular weight species of cystatin C could be found on SDS-PAGE. These results suggest that samples from transgenic mice contain a SDS-unstable high molecular weight cystatin C immunoreactive component (HMWCCIR). It was observed that HMWCCIR was less stable in SDS than the *in vitro* produced oligomers of wt cystatin C (See 6.3.), implying that the aggregates are different. The HMWCCIR could be detected in several tissues, such as the brain, testis, spleen and kidneys, as well as in serum, implying that it is present both intracellularly and extracellularly.

It was observed that papain does not bind to the HMWCCIR, which might suggest that it does not contain correctly folded cystatin C monomers. It could be a complex between cystatin C and a cysteine protease, which also would prevent binding between the HWMCCIR and papain. Another possible explanation is that the HMWCCIR contains domain-swapped species of cystatin C, since a domain-swapped aggregate of cystatin C would not be able to bind to papain (Abrahamson and Grubb 1994; Janowski, Kozak *et al*. 2001).

6.8. Prevention of domain swapping in vitro (Paper I)

Although the production of cystatin C variants stabilized against domain swapping by an extra disulfide bond was useful in demonstrating that prevention of domain swapping inhibits the formation of dimers, oligomers and fibrils, it is obvious that if treatment strategies based upon prevention of domain swapping are to be developed, exogenous agents stabilizing the monomeric form of cystatin C must be sought. Therefore, it was tested whether or not a monoclonal antibody raised against wt cystatin C could inhibit the cystatin C dimerization process. Indeed, the antibody could suppress the dimerization of both wt and L68Q cystatin C. The antibody might react preferentially with "open" cystatin C molecules that are directly involved in the domain swapping process (Fig. 1A). Another mechanism might be that the antibody preferentially reacts with dimeric cystatin C and monomerizes it in the process.

In addition, carboxymethylpapain, an active site-alkylated inert derivative of papain, was observed to inhibit the dimerization of both wt and L68Q cystatin C. It is known that papain, and carboxymethylpapain, strongly bind to the inhibitory centers of monomeric wt and L68Q cystatin C, while no binding occurs with the dimeric protein (See 5.6.3.), suggesting that the stabilizing effect of carboxymethylpapain is due to a stabilization of the monomeric fold of cystatin C. These results indicate, therefore, that it should be feasible to prevent aggregation by stabilizing the monomeric fold of the protein with an exogenous agent.

7. Concluding remarks – Prevention of domain swapping *in vivo*

The observation that different proteins can form pathogenic oligomers and fibrils, with a virtually similar appearance, implies that different protein conformational diseases may have underlying molecular mechanisms in common. One of the proposed mechanisms involved in amyloidosis is domain swapping. This feature has mainly been observed in different crystallized

proteins (Janowski, Kozak *et al*. 2001; Knaus, Morillas *et al*. 2001; Janowski 2004; Janowski, Kozak *et al*. 2005). One could therefore suspect the phenomenon to be an artefact due to crystallization conditions, using high protein concentrations and non-physiological buffers. It has also been suggested that any protein with a free N- or C-terminus has the potential to undergo domain swapping (Liu and Eisenberg 2002).

However, an increasing body of experimental evidence suggests different roles of this mechanism *in vivo*, *e.g.* functional regulation of proteins (Vitagliano, Adinolfi *et al*. 1999). In addition, *in vitro* fibrillization of a variant of T7 endonuclease has provided experimental evidence of propagated domain swapping as a mechanism in the formation of amyloid-like fibrils (Guo and Eisenberg 2006). Furthermore, domain swapping can be relevant in both dimer and fibril formation of the amyloidogenic prion protein (Lee and Eisenberg 2003). In this thesis I have shown that prevention of domain swapping inhibits *in vitro* dimerization, oligomerization and fibrillization of the amyloidogenic protein cystatin C, implying that this mechanism is a prerequisite of aggregation of the protein. Unfortunately, we have not been able to show the relevance of domain swapping *in vivo*. However, it is known that *in vitro* produced dimers of L68Q cystatin C is virtually identical to the domain-swapped dimers of wt cystatin C (Gerhartz, Ekiel *et al*. 1998; Janowski, Kozak *et al*. 2001), implying that also the dimer of L68Q cystatin C is formed by domain swapping. Since cystatin C dimers can only be detected *in vivo* when L68Q cystatin C is expressed, *i.e.* in patients suffering from HCCAA (Bjarnadottir, Nilsson *et al*. 2001), it is not impossible that the mechanism of domain swapping is occurring *in vivo*.

Although amyloid fibrils are very stable, the equilibrium between monomers and fibrils can be influenced *in vivo* (Pepys, Herbert *et al*. 2002; Pepys 2006). However, today there is no treatment to cure amyloidosis. One approach would be to decrease the concentration of the amyloidogenic protein, *e.g.* by inhibiting cleavage of APP to Aß (Dovey, John *et al*. 2001) or by

immunization with anti-amyloid antibodies (DeMattos, Bales *et al*. 2001). Another approach would be to stabilize the monomeric fold of the protein, *e.g.* by preventing domain swapping.

In this thesis, a dimer-suppressing capacity of monoclonal anticystatin C antibodies and carboxymethylpapain have been demonstrated, indicating the feasibility of engineering low molecular mass compounds that might stabilize the native, monomeric form of cystatin C. For example, substances binding to the protease-binding site of cystatin C should be able to stabilize its physiological form. In this thesis it is further shown that the molecular mechanism of domain swapping is involved in many different aggregation step. Hence, if the monomeric fold of the protein can be stabilized, not only dimerization will be inhibited, but probably also oligomerization and fibrillization, which should result in prevention of amyloidosis.

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