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FIBRILLOGENIC OLIGOMERS OF HUMAN CYSTATIN C ARE FORMED BY PROPAGATED DOMAIN SWAPPING*

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Running title: Propagated domain-swapped cystatin C oligomers form fibrils

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Cystatin C and the prion protein have been shown to form dimers via three-dimensional domain swapping and this process has also been hypothesized to be involved in amyloidogenesis. Production of oligomers of other amyloidogenic proteins has been reported to precede fibril formation, suggesting oligomers as intermediates in fibrillogenesis. A variant of cystatin C, with an L68→Q substitution, is highly amyloidogenic and carriers of this mutation suffer from massive cerebral amyloidosis leading to brain hemorrhage and death in early adulthood. This work describes doughnut-shaped oligomers formed by wild type and L68Q cystatin C upon incubation of the monomeric proteins. Purified oligomers of cystatin C are shown to fibrillize faster and at a lower concentration than the monomeric protein, indicating a role of the oligomers as fibril-assembly intermediates. Moreover, the present work demonstrates that three-dimensional domain swapping is involved in the formation of the oligomers, since variants of monomeric cystatin C, stabilized against three-dimensional domain swapping by engineered disulfide bonds, do not produce oligomers upon incubation at non-reducing conditions. Redox experiments using wild type and stabilized cystatin C strongly suggest that the oligomers, and thus probably the fibrils as well, are formed by propagated domain swapping rather than by assembly of domain-swapped cystatin C dimers.

Insoluble amyloid fibrils are found as deposits in patients with a range of diseases, e.g. Alzheimer's disease, Parkinson's disease, reactive amyloidosis, cystatin C amyloidosis, and the prionoses (1-3). Knowledge of the molecular mechanism causing the transition of physiologically normal and soluble proteins to toxic oligomers and insoluble fibrils is essential

for the development of treatment strategies for this group of common, but currently incurable, diseases. Production of oligomers of different amyloidogenic proteins has been reported to precede the fibril formation stage, suggesting oligomers as intermediates in fibrillogenesis (4-6).

Human cystatin C is involved in *in vivo* fibrillogenesis as the commonly occurring normal variant (wt¹ cystatin C) and as a more rare but extremely amyloidogenic L68Q cystatin C variant. Patients suffering from Hereditary Cystatin C Amyloid Angiopathy (HCCAA) have L68Q cystatin C deposited as amyloid fibrils in the cerebral arteries, resulting in cerebral hemorrhage and death in early adulthood (7). In the blood plasma and cerebrospinal fluid of HCCAA patients, high concentrations of non-physiological cystatin C dimers are detected (8). Moreover, wt cystatin C participates in the formation of amyloid deposits together with the A β peptide (1,2). This has been observed particularly in elderly individuals and in patients suffering from Alzheimer's disease or Down's syndrome.

Cystatin C belongs to the cystatin superfamily of reversible inhibitors of cysteine proteases of the papain and legumain families (2). This low molecular mass protein (13 343 Da), expressed in all nucleated human cells, is composed of one polypeptide chain of 120 amino acid residues with two disulfide bonds in its C-terminal part. The general fold of monomeric inhibitors of the cystatin superfamily has been defined by the crystal structure of chicken cystatin (9). It consists of a long α -helix running across a grip of a five-stranded antiparallel β -sheet (Fig. 1A). Several crystal structures of wt cystatin C have been reported so far, but in all of them the protein exists in the form of symmetric three-dimensional domain-swapped dimers (Fig. 1B) (10-12), probably

corresponding to the dimers detected in HCAA patients. In these dimers, tertiary structure elements of the monomeric fold are exchanged between the two participating monomers. The mechanism of three-dimensional domain swapping requires partial unfolding, in which the exchanged domains separate in order to recombine in the oligomeric context. From the time of its definition in diphtheria toxin (14), three-dimensional domain swapping has been observed in more than 30 different proteins, among them in such amyloidogenic proteins as prions (15) and β 2-microglobulin (16,17).

Although both monomeric wt and L68Q cystatin C are known to produce dimers and amyloid fibrils *in vitro*, no high-order oligomeric forms of these proteins have been described. However, such oligomers have been observed for other amyloidogenic proteins, such as A β (4,18,19) and α -synuclein (5,18). The mechanism behind the formation of these oligomers is not well established. Although oligomers have been suggested as productive intermediates in fibrillogenesis, off-pathway oligomers of prion proteins have also been reported (20). The oligomers themselves are probably of pathophysiological significance, since they may be substantially more toxic than the mature amyloid fibrils (19,21).

It has been suggested that the process of three-dimensional domain swapping, when propagated in an open-ended fashion, could be the basis of the formation of amyloid fibrils (22-27). We have earlier reported on stabilized variants of monomeric wt and L68Q cystatin C, with cysteine mutations introduced for selective formation of disulfide bridges between the tertiary structure elements that undergo separation on domain swapping (Fig. 1C) (28). The stabilized variants were found to have a drastically decreased capability to form dimers and fibrils. Although demonstrating the importance of three-dimensional domain swapping in the generation of dimers and fibrils, these observations were not able to determine whether the domain swapping occurred in a propagated way or was limited to the formation of closed-ended dimers. In order to further elucidate the molecular pathophysiological mechanism of the transformation of monomeric wt and L68Q cystatin C into amyloid fibrils, the present work aimed at identifying and characterizing any possible oligomeric intermediates. Indeed, such oligomers could be identified and purified. It was also possible to demonstrate that these oligomers are intermediates in fibrillogenesis, and not off-pathway products. Furthermore, our data clearly

indicate that propagated three-dimensional domain swapping is pivotal in oligomer formation.

EXPERIMENTAL PROCEDURES

Q-Sepharose and Superdex HR75 were from Pharmacia (Uppsala, Sweden). Centrifugal microconcentrators were purchased from Amicon (Bedford, MA, USA). NuPage polyacrylamide gels and Mark12 unstained standard were obtained from Invitrogen (Carlsbad, CA, USA). The silver staining kit was purchased from Bio-Rad (Hercules, CA, USA). Polyclonal rabbit antibodies against wt cystatin C and horse-radish peroxidase-labeled polyclonal pig anti-rabbit antibodies were bought from Dako (Copenhagen, Denmark). Polyclonal rabbit anti-A β oligomer A11 antibodies were purchased from Biosource (Camarillo, USA). Nitrocellulose membranes were obtained from Ancos (Højby, Denmark). Dried milk was bought from Semper (Sundbyberg, Sweden). EZ-ECL chemiluminescence detection kit for horse-radish peroxidase was from Biological Industries (Kibbutz Beit Haemek, Israel). Unless specified, all other chemicals used were of analytical grade and obtained from Sigma (St. Louis, MO, USA).

Protein Expression and Purification - The different cystatin C variants were expressed and purified as previously described (28-31). Briefly, wt cystatin C was expressed in *E. coli* and isolated from periplasmic extracts by a two-step procedure (29,30). A similar procedure was used for the expression of two cystatin C variants, wt cystatin C stab1 (Fig. 1C) and wt cystatin C stab2, stabilized against three-dimensional domain swapping by inserted disulfide bonds (28). The proteins were purified by anion exchange chromatography using Q-Sepharose in 20 mM ethanolamine, pH 9.0, containing 1 mM benzamidine chloride. After concentration, the samples were subjected to gel chromatography using a Pharmacia FPLC Superdex HR75 column and 50 mM ammonium bicarbonate. The proteins were lyophilized and stored at room temperature. In the case of L68Q cystatin C, the protein was purified from inclusion bodies by dissolving them in 6 M guanidinium chloride, followed by refolding of the protein on a Superdex HR75 column in 50 mM ammonium bicarbonate (31). After gel chromatography, L68Q cystatin C was lyophilized and stored at room temperature.

Determination of Protein Concentration - The protein concentration of the solutions of monomeric cystatin C was determined by UV absorption spectroscopy at 280 nm using a molar extinction coefficient of $\epsilon = 11\,100\text{ M}^{-1}\text{ cm}^{-1}$ ($A_{280,0.1\%} = 0.83$) (32). The concentration of oligomeric species was measured using Coomassie Protein Assay Reagent (Pierce, Rockford, USA) and a DPC Milenia spectrophotometer.

Formation of Oligomers and Amyloid Fibrils - Wild type cystatin C and its two variants, stabilized against three-dimensional domain swapping (28), were incubated at a concentration of 3 mg/ml in 50 mM NaAc, 100 mM NaCl, pH 4.0, at 48 °C with continuous agitation. The same incubation conditions were used for L68Q cystatin C, except that a lower protein concentration of 0.6 mg/ml was used. Oligomerization of stabilized monomeric cystatin C variants was induced by addition of DTT to a final concentration of 3.5 mM, corresponding to a 2-3-fold molar excess over the cysteine content of the proteins, followed by 1 h of incubation at 48 °C with continuous agitation, after which the mixtures were extensively dialysed in jars open to the ambient atmosphere.

Purification of Oligomers - Oligomers were purified by repeated pressure ultrafiltration using microconcentrators with a cut-off of 100 kDa. A sample with unpurified oligomers was inserted into a rinsed microconcentrator cell and centrifuged at 9800 rpm. The oligomers retained in the cell were diluted with 50 mM NaAc, 100 mM NaCl, pH 4.0, and ultrafiltered again. The dilution-ultrafiltration cycle was repeated three times. Finally, the volume of the retentate with the oligomers was adjusted with buffer and the solution was transferred to an eppendorf tube by centrifugation at 3000 rpm for 3 min. All centrifugation steps were carried out at 4 °C.

Seeding - Seeds were prepared using a water bath-type ultrasonic transmitter to sonicate preformed mature fibrils of wt and L68Q cystatin C for two times 30 min. Aliquots of the suspensions of seeds were added to the starting solution containing 3 mg/ml of monomeric wt cystatin C in 50 mM NaAc, 100 mM NaCl, pH 4.0. When the seeding capacity of oligomers was tested, oligomers were purified as described above and added to the starting solution.

Electron Microscopy - Protein samples (5 μ l) were applied on a glow-discharged carbon-

coated copper grid (400 mesh). After 1 min of adsorption, excess liquid was removed using filter paper and the samples were negatively stained with 2% (w/v) aqueous uranyl acetate for 30 sec. The samples were examined with a Philips CM10 electron microscope (Philips, Eindhoven, The Netherlands), operating at an excitation voltage of 60 or 100 kV. Electron micrographs were analyzed using the software Image J (33).

Quantitative Estimation of Amyloid Fibrils - The formation of amyloid fibrils was estimated by the thioflavine T (ThT) fluorescence assay as described by LeVine (34,35). Aliquots were removed from the sample at timed intervals and analyzed. Protein samples were diluted with 50 mM glycine-sodium hydroxide buffer, pH 8.5, containing 100 μ M ThT. Immediately after dilution, measurements were carried out in black polystyrene 96 well microtiter plates (Nunc F96 MicroWell Plates, Roskilde, Denmark), pretreated with Sigmacote (Sigma). The fluorescence was recorded using a FLUOstar OPTIMA fluorimeter at excitation and emission wavelengths of 450 and 490 nm, respectively. When the fibrillization capacity of purified oligomers was tested, the change in fluorescence intensity for the incubated samples was calculated with reference to the fluorescence intensity of the starting material. The significance of fluorescence intensity differences was tested by the non-parametric Wilcoxon rank sum test.

Electrophoresis - Oligomers were visualized by two SDS-PAGE systems, using pre-cast NuPage Novex Bis-Tris gradient gels (4-12%) and NuPage Novex Tris-Acetate gradient gels (3-8%). The Bis-Tris gels were run with the morpholinoethansulfonate buffer system, pH 6.4, at 200V for 35 min. The samples were applied with a loading buffer containing a final concentration of 10% (w/v) glycerol, 141 mM Tris base, 106 mM Tris-HCl, 0.1% (w/v) SDS, 0.51 mM EDTA, and 0.075% (w/v) ServaBlue. The Tris-Acetate polyacrylamide gels were run in a buffer of 1.1% (w/v) β -alanine, 1% (v/v) HAc, 0.1% (w/v) SDS and with use of a loading buffer of 10% (w/v) glycerol, 1.1% (w/v) β -alanine, 1% (v/v) HAc, 0.1% (w/v) SDS, and 0.075% (w/v) ServaBlue. The Tris-Acetate gels were run at 150V during 1 h. The gels were silver stained as described by the manufacturer.

Circular Dichroism (CD) Measurements - Fibrils and purified oligomers of wt cystatin C were prepared in 50 mM NaAc, 100 mM NaCl,

pH 4.0. Lyophilized monomeric wt cystatin C was dissolved in 10 mM NaAc, pH 4.0. Prior to use, the stock solution of each form of cystatin C was diluted in 10 mM NaAc, pH 4.0, to a final maximal concentration of approximately 0.2 mg/ml. Two hundred μ l of the various protein solutions were placed in a cuvette with 1 mm path length, and CD spectra were collected on a Jasco J-810 spectropolarimeter at 20 °C. A CD spectrum of the buffer was subtracted from each sample spectrum for background correction. Spectra were recorded using a step size of 1 nm and a bandwidth of 0.4 nm. Ten consecutive scans were recorded for all spectra on average. The stock solution of each form of cystatin C was serially diluted and scans were recorded for each dilution until no signal due to the protein could be obtained.

Immunoblotting - Dot-blotting using nitrocellulose membranes and polyclonal rabbit anti-cystatin C and anti-oligomer A11 antibodies was performed as described by the manufacturer of the anti-oligomeric antibodies, Biosource. Briefly, 1-3 μ l of the protein samples was applied on a nitrocellulose membrane and air-dried. The membrane was then blocked by the use of a solution of 10% (w/v) milk proteins, washed, and incubated with the primary antibody for 1 h. After further washing, the membrane was incubated with the secondary antibody for 45 min and developed using the EZ-ECL chemiluminescence detection kit as described by the manufacturer.

RESULTS

In Vitro Fibril Formation of wt and L68Q Cystatin C and wt Cystatin C Stabilized against Domain Swapping - We have previously shown that recombinant wt cystatin C forms amyloid fibrils *in vitro* in solutions at pH 2.0 (28). In order to produce fibrils at more physiological conditions and at a slower rate allowing detection of possible oligomers, we have selected a concentration of 3 mg/ml of wt cystatin C, and incubation with continuous agitation at 48 °C in 50 mM NaAc, 100 mM NaCl, pH 4.0. An exponential increase in the ThT fluorescence intensity, indicating the formation of fibrils, was observed after 3 weeks of incubation (Fig. 2A). Electron micrographs at this stage showed long, non-branched, twisted fibrillar structures (Fig. 2B). The diameter of the mature fibrils of wt cystatin C was 10.4 ± 2.2 nm (mean \pm SD; n=200).

The propensity of L68Q cystatin C to form fibrils *in vitro* has not been tested so far

due to difficulties in producing sufficient amounts of recombinant L68Q cystatin C. In the present investigation, we used a protein concentration of 0.6 rather than 3 mg/ml in the incubation mixture. L68Q cystatin C was observed to form fibrils even at this reduced protein concentration and with a lag phase of less than 3 days (Fig. 2A). The L68Q cystatin C fibrils were similar in appearance to those of wt cystatin C, but showed a higher tendency for lateral attachment (Fig. 2C). The diameter of the L68Q cystatin C fibrils was 11.1 ± 2.3 nm (n=148).

Incubation at a concentration of 3 mg/ml of a variant of wt cystatin C (wt cystatin C stab1), stabilized against three-dimensional domain swapping, did not result in any fibril formation during the entire incubation period of 38 days (Fig. 2A).

In Vitro Oligomer Formation of wt and L68Q Cystatin C - In order to detect any possible intermediates preceding the formation of amyloid fibrils, we closely examined the incubated samples during the lag phase, when no fibrils are present in the solution, as estimated by the ThT fluorescence assay. Most studies employed wt cystatin C incubated at a concentration of 3 mg/ml. During the lag phase, starting already at 1 h of incubation, electron microscopy demonstrated doughnut-shaped objects with a central hole (Fig. 3A, B). No fibrils could be detected by electron microscopy until the exponential phase of the increase in ThT fluorescence. The oligomer populations were rather homogenous, with a Gaussian size distribution. The mean outer diameter was 13.4 ± 1.9 nm (n=916) and the mean inner diameter was 2.7 ± 0.7 nm (n=176). The mean width of the ring was 5.4 ± 0.9 nm (n=192). Incubation of L68Q cystatin C at 0.6 mg/ml also produced doughnut-shaped oligomers with virtually identical appearance in electron microscopy (Fig. 3C).

The oligomers were also analyzed using two different SDS-PAGE systems. Both systems detected oligomers of wt cystatin C already after 1 h of incubation. The oligomers could be observed as a smear corresponding to a molecular mass of 200 - 300 kDa (data not shown).

Purification of Cystatin C Oligomers - Despite many attempts, we failed to purify the oligomers by size exclusion chromatography. However, repeated pressure ultrafiltration using filter cells with a cut-off of 100 kDa allowed a virtually complete separation of the oligomers from the

monomeric species as assessed by SDS-PAGE. The retentate, containing the oligomers, was diluted and ultrafiltered three times using 100 μ l of buffer each time. As can be seen in Fig. 4A, the last filtrate did not contain any detectable amount of protein, whereas the oligomers are present in the retentate. These observations were corroborated by electron microscopy, showing doughnut-shaped objects in the retentate, but not in the filtrate. The purified oligomers had the same appearance as those in the original solution (Fig. 3A, B and 4B).

Cystatin C Oligomers Do Not Show Any Seeding Effect – Seeding a solution of an amyloidogenic protein with sonicated preformed amyloid fibrils reduces the lag phase for fibril formation significantly, possibly by the elimination of a nucleation step (36). Indeed, when sonicated preformed fibrils of either wt or L68Q cystatin C were added to the monomeric starting solution, the lag phase of wt cystatin C fibrillization was reduced by more than two weeks, even at very low concentrations of added sonicated fibrils. In contrast, addition of different concentrations of purified oligomers of wt cystatin C up to approximately 2% of the starting cystatin C concentration, did not significantly shorten the lag phase.

The Structure of Cystatin C Oligomers Is Related to that of Oligomers of Other Amyloidogenic Proteins - Using polyclonal antibodies raised against A β oligomers, Kaye *et al.* have found that soluble oligomers of several different proteins display a common structure regardless of the amino acid sequences of the proteins (37). We used the anti-A β oligomer antibody A11 and found that it reacts with wt cystatin C oligomers, but not with monomeric cystatin C used as starting material for the production of oligomers (Fig. 5).

Domain Swapping Is a Prerequisite for Oligomerization and Fibrillization - The crystal structure of dimeric wt cystatin C demonstrates that the dimer is formed via three-dimensional swapping of domains between the two participating monomers (10-12). We have earlier reported on stabilized variants of monomeric wt cystatin C (called wt cystatin C stab1 and wt cystatin C stab2) with cysteine mutations introduced for selective formation of disulfide bridges between the tertiary structure elements that must undergo separation in an unfolding event preceding three-dimensional domain swapping (Fig. 1C) (28). The stabilized variants were found to have a drastically decreased

capability to produce dimers and amyloid fibrils at pH 2.0. In accordance with those earlier results, wt cystatin C, but not the stabilized variants, was found to produce fibrils at pH 4.0 as tested both by the ThT fluorescence assay and by electron microscopy (Fig. 2A, B). After 3 weeks of incubation, a substantial amount of fibrils could be observed in the solution of wt cystatin C, while no fibrils could be detected in the solutions of wt cystatin C stab1 (Fig. 2A) or wt cystatin C stab2 (data not shown). Even a prolongation of the incubation period up to 38 days failed to generate amyloid fibrils from the stabilized variants. Repeated analysis by electron microscopy and SDS-PAGE demonstrated the production of oligomers from wt cystatin C already after 1 h of incubation, whereas the stabilized variants did not produce any oligomers during the entire observation period of 168 h. In addition, electron microscopy of the solution of wt cystatin C stab1 incubated for 38 days did not detect any oligomers.

Evidence for Propagated Domain Swapping in Cystatin C Oligomerization - The increased intrinsic stability of wt cystatin C stab1 and wt cystatin C stab2 is due to an inserted extra disulfide bond, joining the structural elements of wt cystatin C involved in three-dimensional domain swapping (Fig. 1C) (28). Reduction of this disulfide bond will, therefore, abrogate the stability of wt cystatin C stab1 and wt cystatin C stab2 and, furthermore, will allow interaction between the generated cysteinyl SH-groups of separate cystatin C monomers. Redox experiments were therefore performed to further elucidate the role of three-dimensional domain swapping in the oligomerization process. As reported above, incubation of wt cystatin C stab1 and wt cystatin C stab2 in the absence of reducing agents, did not produce any oligomers. However, when the stabilized cystatin C variants were incubated in the presence of the reducing agent DTT, oligomers could be observed within 1 h, showing up as a high molecular mass band on SDS-PAGE (Fig. 6; lane 4) and as doughnut-shaped objects, 13 nm in diameter, in electron micrographs (Fig. 3D). After removal of DTT by dialysis, the stability of the formed oligomers was examined using SDS-PAGE (Fig. 6). Purified wt cystatin C oligomers are stable in 0.1% SDS, but dissociate into monomers if the SDS concentration is raised to 2%. In contrast, the major fraction of the oligomers produced upon incubation of wt cystatin C stab1 or wt cystatin C stab2 at reducing conditions followed by extensive dialysis, is still stable in the presence of 2% SDS. However, also these

oligomers dissociate into monomers upon addition of 50 mM DTT (Fig. 6).

CD Measurements – The purified oligomers of wt cystatin C were observed to weakly interact with ThT, suggesting the presence of an ordered β -structure (data not shown). To test whether or not the oligomers were enriched in β -sheet structure, CD measurements were performed for monomers, purified oligomers, and fibrils of wt cystatin C (Fig. 7). Spectra of serial dilutions of the stock solutions of each form of cystatin C were recorded. No changes in the wavelengths of the minima of the spectra could be observed indicating concentration-independent spectra. The CD spectra indicated a shift in secondary structure, consistent with a gradual increase in β -structure concomitant with oligomerization and fibril formation.

Purified Wild Type Cystatin C Oligomers Form Fibrils - In order to investigate whether the oligomers of wt cystatin C are indeed fibril-assembly intermediates and not off-pathway products, purified oligomers were incubated in 50 mM NaAc, 100 mM NaCl, pH 4.0, at 48 °C with continuous agitation. The fibrillization rate was tested at a much lower concentration of the oligomers (approximately 0.03 mg/ml) than that used previously for the production of fibrils from monomeric wt cystatin C (3 mg/ml). Nevertheless, the purified oligomers were repeatedly observed to fibrillize upon incubation, as tested both by the ThT fluorescence assay and by electron microscopy (Fig. 8). The diameter of the fibrils was 11.2 ± 2.3 nm ($n=150$), which agrees with that of the fibrils generated from monomeric wt cystatin C, incubated at a concentration of 3 mg/ml, as well as that of the fibrils generated from L68Q cystatin C, incubated at a concentration of 0.6 mg/ml. However, the fibrils generated from the oligomers showed a tendency for lateral attachment, like the fibrils generated from L68Q cystatin C. In simultaneous experiments, monomeric wt cystatin C was incubated in concentrations of 0.03 and 0.3 mg/ml, but no fibrils could be detected during the observation period of 13 days (Fig. 8).

DISCUSSION

Different molecular mechanisms for the transition of physiologically normal and soluble proteins to oligomers and insoluble amyloid fibrils have been proposed (3, 36, 38-39). A comprehensive knowledge of the molecular pathophysiology of the production of amyloid fibrils and oligomers includes determination of

the structures of all intermediates in the transformation process and understanding the ways in which one intermediate is transformed into the next. Such knowledge will provide a rational basis for the development of new diagnostic and treatment options. Slowing down the formation of a toxic intermediate or accelerating its transformation into another, perhaps less toxic, intermediate might, for example, reduce the accumulation of the toxic intermediate. The present study is concerned with the detection, characterization and mechanism of formation of fibril-assembly intermediates of human cystatin C.

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 (8). 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 oligomerization pathway (10,22). L68Q cystatin C, the cystatin C variant causing hereditary cystatin C amyloid angiopathy, has an increased propensity to form dimers compared to that of wt cystatin C, since the mutation lowers the energy barrier for the transition through destabilization of the monomeric structure and stabilization of the unfolded intermediate (22). However, also wt cystatin C has been shown to dimerize *in vitro* and the rate of the process could be increased by raising the temperature, lowering the pH, or using conditions of mild chemical denaturation (40). Transformation of monomeric wt cystatin C into amyloid fibrils *in vitro* has also been described, but the system used required a very low pH of 2.0 and did not allow detection of any molecular intermediates of the transformation (28).

In the present work, less drastic conditions for the production of cystatin C amyloid fibrils have been used in order to allow detection of intermediates that might be stable at physiological conditions but not at pH 2.0. To this end, a suitable incubation system at pH 4.0 was found and in this system monomeric wt cystatin C, at a concentration of 3 mg/ml, produced amyloid fibrils after about 3 weeks. The propensity of L68Q cystatin C to form fibrils *in vitro* has not been tested so far due to difficulties in producing sufficient amounts of recombinant protein. However, incubation of L68Q cystatin C at the presently described conditions at a concentration of 0.6 mg/ml, resulted in the production of amyloid fibrils within only three days. This indicates that the substitution of glutamine for leucine in L68Q

cystatin C not only results in an enhanced dimerization rate of the molecule *in vitro*, but also in an increased propensity to form amyloid fibrils. Electron microscopy showed that the L68Q cystatin C fibrils were similar in appearance to those of wt cystatin C but displayed a tendency to interconnect laterally, forming large clusters of fibrils. A similar type of lateral attachment has been observed in samples of proteinase-K-digested prion fibrils (41).

To demonstrate oligomer formation prior to fibrillization of human cystatin C, we used several analytical systems. Monitoring the incubation solutions before the formation of amyloid fibrils by electron microscopy revealed the presence of oligomers already after 1 h of incubation. 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 is remarkably similar to that described for oligomers of other amyloidogenic proteins. Annular structures with a central depression have previously been detected early in the fibrillization process of *e.g.* α -synuclein (5) and A β (4). The oligomers of α -synuclein had an outer diameter of 8-12 nm and an inner diameter of 2.0-2.5 nm, and those of a variant of A β had an outer diameter of 7-10 nm and an inner diameter of 1.5-2.0 nm (18). These results support the notion that, like the fibrils (42), 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, Kaye *et al.* found that oligomers of several different proteins all bound to polyclonal antibodies raised against A β oligomers, whereas those antibodies did not bind to the native monomeric proteins (37). Indeed, these oligomer-specific antibodies also bound to the oligomers of wt cystatin C, but not to monomeric or dimeric cystatin C.

Two different SDS-PAGE systems could also be used to demonstrate the early appearance of cystatin C oligomers in the incubation mixtures used to produce amyloid fibrils. The oligomers could be observed as a smear, representing molecular species with a mass between 200 and 300 kDa. The smears observed in the electropherograms suggest a higher heterogeneity of the oligomeric populations than would be suggested by the

uniformity of the oligomeric populations observed in the electron micrographs. However, the oligomers observed in the electron micrographs might be of varying heights, and thus possess varying molecular mass, since it is not possible to distinguish between small differences in height with the negative staining technique used in the present work.

Despite many attempts, we failed to purify the oligomers of cystatin C by size exclusion chromatography. However, repeated pressure ultrafiltration, using filters with a cut-off of 100 kDa, allowed a successful separation of the oligomers from the monomers as assessed by electron microscopy and SDS-PAGE. The final retentate contained virtually only oligomers, whereas the filtrate of the first ultrafiltration cycle contained monomers but no oligomers. The doughnut-shaped oligomers identified by electron microscopy in the final retentate had the same appearance as those in the incubation solution before purification. A similar procedure of purification of oligomers from low molecular weight species has been reported for A β (43).

Crystallographic studies of two amyloidogenic proteins, the human prion protein and cystatin C, have shown that they form dimers by three-dimensional domain swapping, *i.e.* by reciprocal replacement of an element of the tertiary structure of one molecule with an identical element from the other protein molecule (Fig. 1B) (10-12,14,15). We have earlier reported on stabilized variants of monomeric wt cystatin C with cysteine mutations introduced for selective formation of disulfide bridges between the tertiary structure elements that undergo separation on unfolding and exchange in three-dimensional domain swapping (Fig. 1C) (28). The stabilized variants were found to have drastically decreased capabilities to form dimers and fibrils. In order to investigate the role of three-dimensional domain swapping in the oligomerization process, the capacity of the stabilized wt cystatin C variants to oligomerize has been tested in the present study. Incubation of the stabilized variants of monomeric wt cystatin C at conditions producing large amounts of oligomers from unaltered wt cystatin C did not result in any detectable production of oligomers. Prevention of three-dimensional domain swapping, therefore, seems to suppress the formation of oligomers, indicating the importance of three-dimensional domain swapping also for oligomerization of cystatin C.

The experimental data available to date do not elucidate the question of whether the

formation of oligomers or fibrils involves closed-ended domain-swapped cystatin C dimers as the building blocks, or whether propagated domain swapping is involved. Propagated (or run-away) domain swapping refers to a process whereby a protein molecule swaps a domain into a complementary domain site of a second identical protein molecule, which in its turn swaps an identical domain into the complementary site of a third protein molecule etc., resulting in an open-ended chain of intertwined identical protein subunits (Fig. 1C) (10,15,22,27,44). A recent study of *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 (25). To investigate a possible role of propagated three-dimensional domain swapping in the formation of cystatin C oligomers, redox experiments have been performed using stabilized wt cystatin C variants. The intrinsic stability of the stabilized cystatin C monomers is due to an inserted extra disulfide bond, joining the structural elements involved in three-dimensional domain swapping (28). Reduction of this disulfide bond will therefore abrogate the stability of the monomeric proteins and, furthermore, will enable disulfide bond formation between the generated cysteinyl SH-groups of separate cystatin C monomers upon reversal of the environment to non-reducing conditions. After reduction, the stabilized variants should, therefore, be as prone to oligomerization and fibrillization 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 within 1 h of incubation, as assessed by electron microscopy and SDS-PAGE. The apparent size of the oligomers in SDS-PAGE was similar to that observed for wt cystatin C. Electron microscopy showed doughnut-shaped objects, 13.4 nm in diameter, but other types of aggregates could also be observed. The oligomers formed from wt cystatin C at non-reducing conditions are stable in 0.1% SDS, but dissociate into monomers in higher SDS concentrations. In contrast, the oligomers produced upon incubation of the stabilized variants at reducing conditions followed by removal of the reducing agent by extensive dialysis, are stable in 2% SDS. However, reduction of the oligomers stable in 2% SDS resulted in their complete dissociation into monomers. These results are compatible with the formation of a chain of cystatin C molecules linked by propagated domain

swapping and stabilized by disulfide bonds between the swapped domains of adjacent protein molecules (Fig. 1C).

The oligomers produced from cystatin C might be either fibril-assembly intermediates or off-pathway products. We, therefore, tested whether incubation of purified oligomers would result in fibril formation. Since only a small amount of oligomers was available, their fibrillization capacity was tested at a much lower concentration (approximately 0.03 mg/ml) than that used in fibrillization experiments involving monomeric wt cystatin C (3 mg/ml). Nevertheless, the oligomers were observed to fibrillize, whereas no fibrils could be detected on incubation of solutions containing 0.03 or 0.3 mg/ml of monomeric cystatin C. The fibrillization capacity of the cystatin C oligomers, therefore, seems to be much higher than that of monomeric cystatin C, indicating that the oligomers, indeed, are fibril-assembly intermediates and not off-pathway products. This notion was supported by CD measurements indicating that the amount of β -sheet structure in the oligomers was intermediate compared to the amounts in cystatin C monomers and fibrils.

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 hollow protofibrils or mature fibrils. This would be in agreement with observations suggesting a hollow core of amyloid fibrils (45,46). In this model, propagated three-dimensional domain swapping would contribute to the formation of each oligomeric subunit, but not necessarily to the stacking of the subunits along the fibril axis. However, as wt cystatin C fibrils are about 10.4 nm in diameter, while the oligomers are 13.4 nm wide, some kind of structural rearrangement would be required in the final stages of the fibril formation. 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 (10.4 nm). The observed width of the oligomer rings (5.4 nm) and the helical twist of the fibrils, evident from the electron micrographs, would speak in favour of this model. The short rod-like protofibrils would display sticky ends and recognize each other with high affinity, contributing thereby to the elongation step and resulting in mature fibrils. Propagated three-dimensional 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.

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FOOTNOTES

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¹ The abbreviations used are: A β , amyloid β peptide; CD, circular dichroism; DTT, dithiothreitol; HCCAA, Hereditary cystatin C amyloid angiopathy; SD, standard deviation; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ThT, thioflavine T; wt, wild type.

FIGURE LEGENDS

Fig. 1. The structure and oligomerization of human cystatin C. (A) The structure of monomeric wt human cystatin C deduced from the known structures of chicken cystatin (9) and dimeric wt cystatin C (10). (B) The structure of dimeric wt cystatin C (PDB code 1TIJ). (C) The wt cystatin C stab1 variant is stabilized against domain swapping at non-reducing conditions by a disulfide bridge between the tertiary structure elements that undergo separation on three-dimensional domain swapping. The L47C/G69C mutations and the C47-C69 disulfide bond are modeled and shown in a ball-and-stick representation. At reducing conditions the C47-C69 disulfide bond is broken and the monomer can undergo partial unfolding. Aggregation by propagated three-dimensional domain swapping followed by transfer to non-reducing conditions result in high-order oligomers stabilized by intermolecular C47-C69 disulfide bonds. The model presented in (C) is based upon redox experiments with wt cystatin C stab1. The figure was prepared in DINO (www.dino3d.org) after model building in Xfit (13).

Fig. 2. Amyloid fibril formation of wt cystatin C, L68Q cystatin C and wt cystatin C stab1. (A) Fibrils produced from 3 mg/ml of wt cystatin C (■), 0.6 mg/ml of L68Q cystatin C (○) and 3 mg/ml of wt cystatin C stab1 (□) incubated for various periods in 50 mM NaAc, 100 mM NaCl, pH 4.0, with continuous agitation at 48 °C, were quantified by the thioflavine T (ThT) fluorescence assay. Panels B and C show electron micrographs, at different magnifications, of wt cystatin C fibrils (B) and L68Q cystatin C fibrils (C) produced from solutions incubated for 22 days. Bars representing 100 nm are used as indicators of size.

Fig. 3. Electron micrographs of cystatin C oligomers. (A) Oligomers produced by incubation for 2 days of wt cystatin C (3 mg/ml) in 50 mM NaAc, 100 mM NaCl, pH 4.0, with continuous agitation at 48 °C. A bar representing 100 nm is used as indicator of size. Panels B-D (20x20 nm) show higher magnifications of oligomers of wt cystatin C (B), L68Q cystatin C (C) and wt cystatin C stab1 (D). Incubation at reducing conditions with 3.5 mM DTT was required to induce oligomer formation of wt cystatin C stab1.

Fig. 4. Purification of cystatin C oligomers. (A) Repeated pressure ultrafiltration was used for the purification of the oligomers and a Bis-Tris SDS-PAGE system with silver staining to monitor the procedure. The starting sample was inserted into a microconcentrator filter cell and centrifuged. The filtrate did not contain any oligomers (Filtrate I). The oligomers retained in the filter cell were diluted and the procedure repeated three times (Filtrate II-IV). The final retentate contained the purified oligomers. (B) Electron micrograph of the purified oligomers. A bar representing 100 nm is used as indicator of size.

Fig. 5. Immunoblotting showing that cystatin C oligomers have a structure related to that of oligomers of other amyloidogenic proteins. A solution of monomeric wt cystatin C was incubated to produce cystatin C oligomers which were isolated by repeated ultrafiltration as shown in Fig. 4. Both the purified oligomers in the retentate and those in the original solution reacted with the anti-oligomer antibody A11, raised against A β oligomers. In contrast, the antibody bound neither to monomeric cystatin C in Filtrate I (Fig. 4A), nor to buffer components. Polyclonal antibodies raised against monomeric cystatin C bound to components of the oligomer solutions as well as to monomeric cystatin C in Filtrate I, but not to buffer components.

Fig. 6. SDS-PAGE indicating propagated three-dimensional domain swapping in the formation of cystatin C oligomers. Wild type cystatin C oligomers (wt) are stable in 0.1% SDS, but dissociate into monomers in 2% SDS. When cystatin C variants, stabilized against three-dimensional domain swapping by an extra intramolecular disulfide bond, are incubated at 48 °C in the presence of the reducing agent DTT, oligomers can be observed within one hour. After removal of DTT by dialysis, the stability of the formed oligomers (stab) was examined and they were found to be stable in both 0.1 and 2% SDS at non-reducing conditions (-DTT), but dissociated into monomers upon reduction (+DTT). The samples were analyzed using a Bis-Tris SDS-PAGE system with silver staining. Mark12 Unstained Standard (first lane) is used to indicate molecular mass.

Fig 7. CD spectra of monomers, oligomers, and fibrils of wt cystatin C. The shift in negative ellipticity towards 220 nm indicates a gradual increase in β -sheet structure when monomers (■) are transformed into oligomers (□) and fibrils (▣).

Fig 8. Demonstration of amyloid fibrils generated from purified wt cystatin C oligomers. Fibrils produced from 0.03 mg/ml of oligomeric wt cystatin C (black), 0.3 mg/ml of monomeric wt cystatin C (white) and 0.03 mg/ml of monomeric wt cystatin C (gray) incubated in 50 mM NaAc, 100 mM NaCl, pH 4.0, with continuous agitation at 48 °C, for 0, 7 and 13 days were quantified by the thioflavine T (ThT) fluorescence assay. The change in ThT fluorescence intensity of the incubated samples was calculated relative to the fluorescence intensity of the starting material. The increase in fluorescence for the solution of oligomeric cystatin C was significant for both incubation periods, but not for the solutions of monomeric wt cystatin C. The *inset* above the graph shows an electron micrograph of the fibrils produced after 13 days of incubation of oligomeric wt cystatin C. A bar representing 100 nm is used as indicator of size.

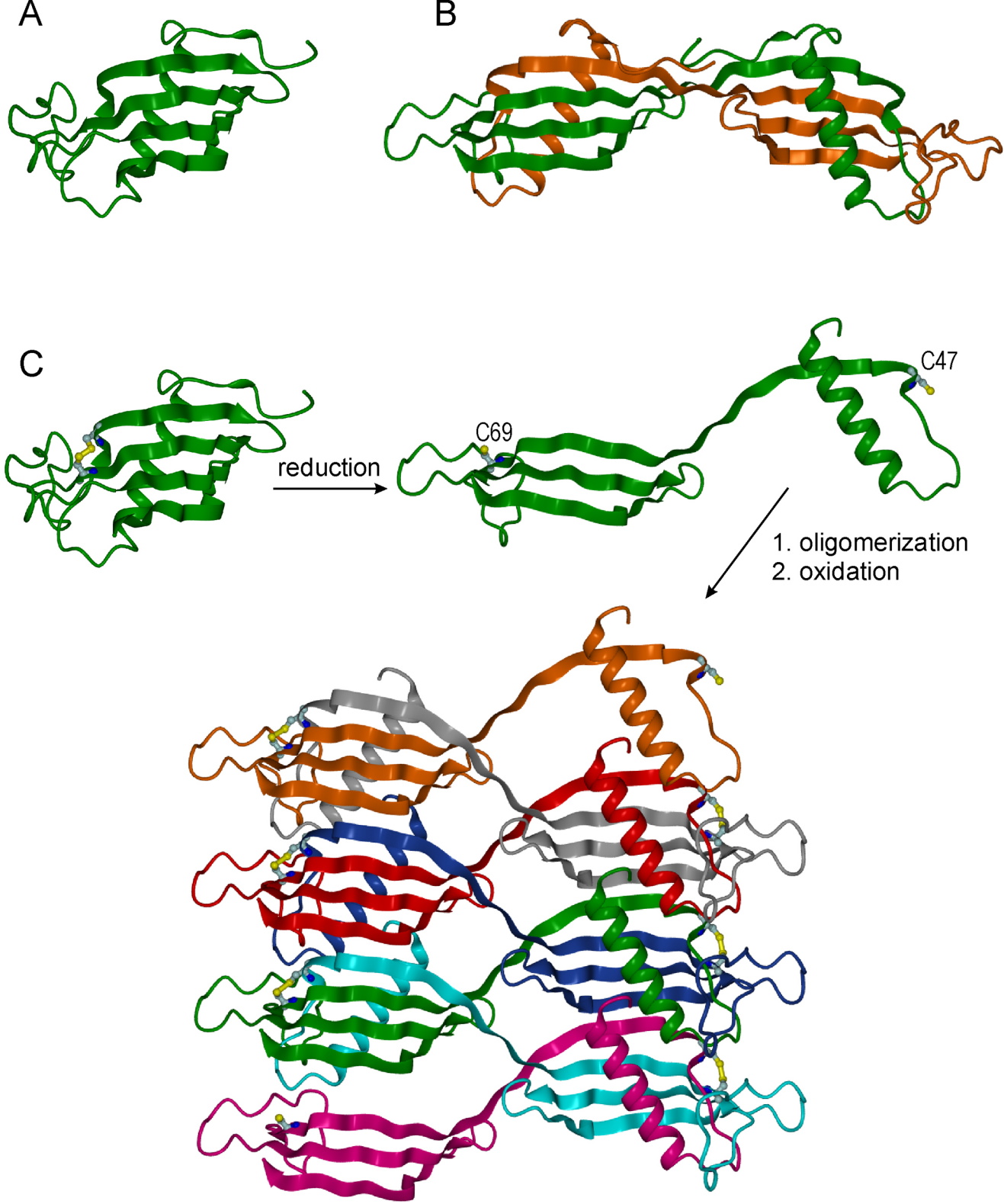


Figure 1

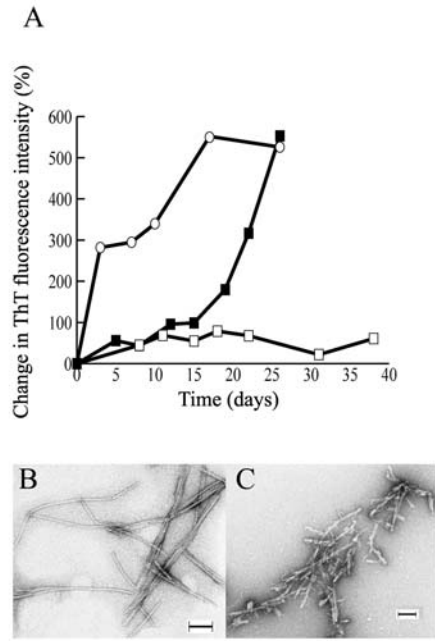


Figure 2

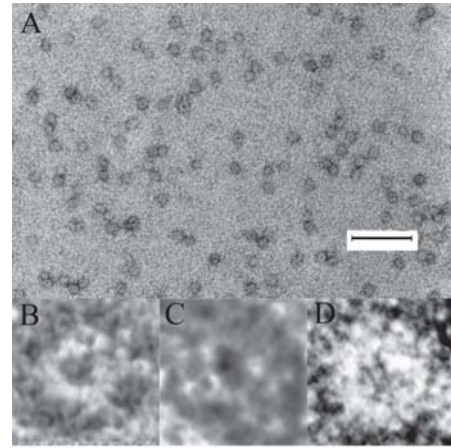


Figure 3

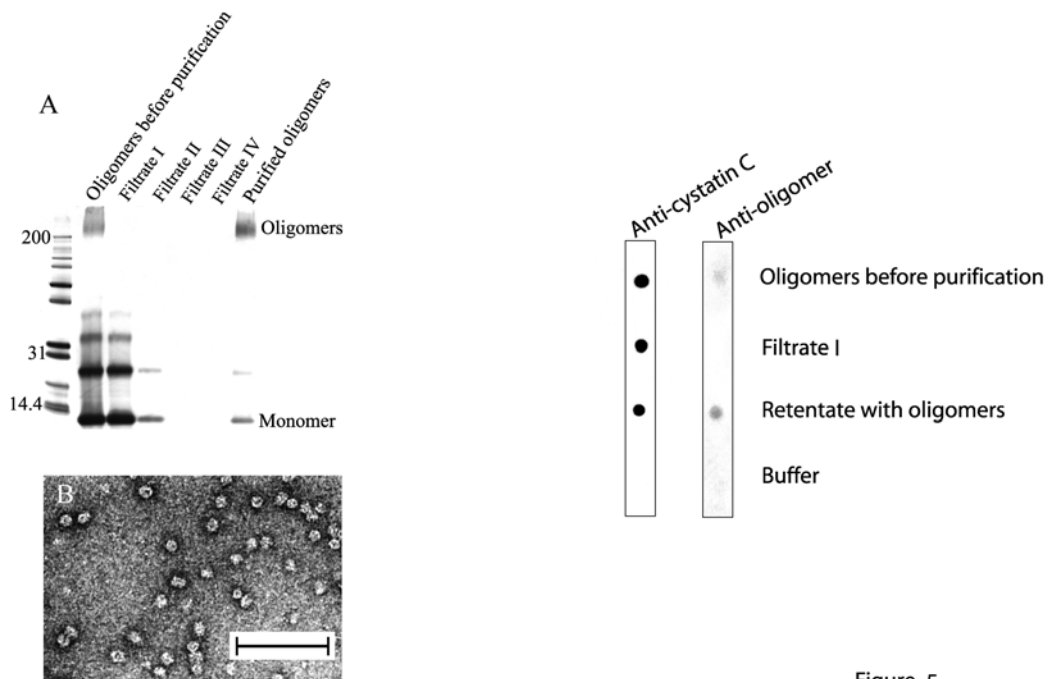


Figure 4

Figure 5

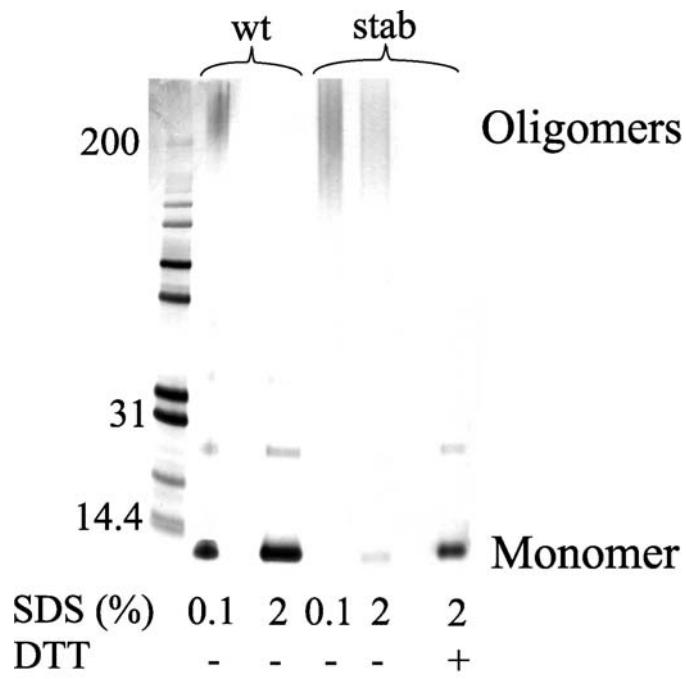


Figure 6

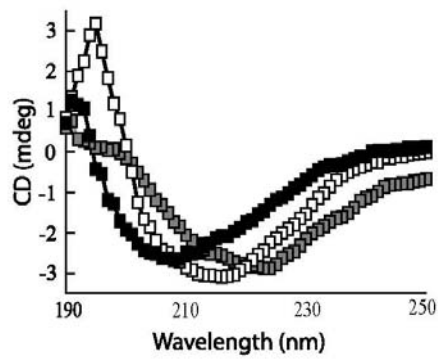


Figure 7

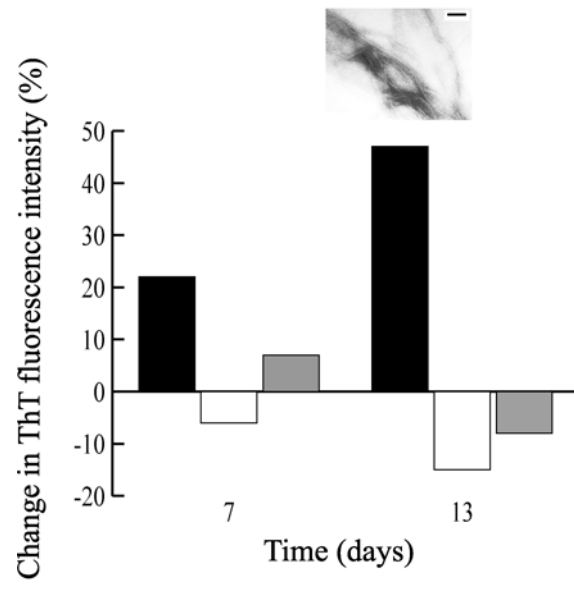


Figure 8