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

Wahlbom, Maria; Wang, Xin; Rodziewicz-Motowidlo, Sylwia; Janowski, Robert; Lindström, Veronica; Önnerfjord, Patrik; Westermark, Gunilla; Grzonka, Zbigniew; Jaskolski, Mariusz; Grubb, Anders

Published in:
Journal of Biological Chemistry

DOI: 10.1074/jbc.M402621200
2004

Link to publication

Citation for published version (APA):

Total number of authors: 10
Prevention of Domain Swapping Inhibits Dimerization and Amyloid Fibril Formation of Cystatin C

USE OF ENGINEERED DISULFIDE BRIDGES, ANTIBODIES, AND CARBOXYMETHYL-PAPAIN TO STABILIZE THE MONOMERIC FORM OF CYSTATIN C*

Received for publication, March 8, 2004, and in revised form, March 16, 2004 Published, JBC Papers in Press, March 17, 2004, DOI 10.1074/jbc.M402621200

Maria Nilsson‡‡, Xin Wang‡‡, Sylwia Rodziewicz-Motowidło‡, Robert Janowski‡, Veronica Lindström‡, Patrik Önnerfjord**, Gunilla Westermark+++ Zbigniew Grzonka†, Mariusz Jaskolski†, and Anders Grubb‡‡‡

From the ‡Department of Clinical Chemistry, University Hospital, S-22185 Lund, Sweden, the **Department of Cell and Molecular Biology, University of Lund, S-22184 Lund, Sweden, the †Department of Organic Chemistry, Faculty of Chemistry, University of Gdańsk, J. Sobieskiego 18, 80-952 Gdańsk, Poland, the ‡‡Department of Cell Biology, Linköping University, S-58183 Linköping, Sweden, and the ‡‡‡Department of Crystallography, Faculty of Chemistry, A. Mickiewicz University and Center for Biocrystallographic Research, Institute of Bioorganic Chemistry, Polish Academy of Sciences, Grunwaldzka 6, 80-790 Poznan, Poland

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 “three-dimensional domain swapping,” has also been suggested to play a part in the generation 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. The present work describes the production of two variants of wild type and L68Q cystatin C with disulfide bridges at positions selected to inhibit domain swapping without affecting the biological function of the four cystatin C variants as cysteine protease inhibitors. The capacity of the four variant proteins to form dimers was tested and compared with that of wild type and L68Q cystatin C. In contrast to the latter two proteins, all four protein variants stabilized by disulfide bridges were resistant toward the formation of dimers. The capacity of the two stabilized variants of wild type cystatin C to form amyloid fibrils was investigated and found to be reduced by 80% compared with that of wild type cystatin C. In an effort to investigate whether exogenous agents could also suppress the formation of dimers of wild type and L68Q cystatin C, a monoclonal antibody or carboxymethylpapain, an inactivated form of a cysteine protease, was added to systems inducing dimerization of wild type and L68Q cystatin C. It was observed that catalytic amounts of both the monoclonal antibody and carboxymethylpapain could suppress dimerization.

Human cystatin C is a low molecular mass protein (13,443 Da, 120 amino acids) belonging to the cystatin superfamily of reversible inhibitors of cysteine proteases of the papain and legumin families (1). Cystatin C is produced by all nucleated human cells and is present in all body fluids (1). Human cystatin C plays an important role in the development of cerebral hemorrhage in patients with a hereditary form of amyloid angiopathy (hereditary cystatin C amyloid angiopathy), where a leucine 68 to glutamine variant of cystatin C (L68Q cystatin C) is deposited as amyloid fibrils in the cerebral arteries, resulting in brain hemorrhage and death in young adults (2). The commonly occurring normal variant of cystatin C (wt cystatin C) also participates in the formation of amyloid depositions together with the amyloid β peptide (Aβ), particularly in elderly individuals and in patients suffering from Alzheimer’s disease or Down’s syndrome (2, 3). Whereas L68Q cystatin C undergoes dimerization and oligomerization in vitro at the temperature of the human body, and is also found in dimeric form in vivo, wt cystatin C shows higher stability (4, 5). However, wt cystatin C can also be dimerized in vitro at elevated temperature, at low pH, and at conditions of mild chemical denaturation (6). The crystal structure of wt cystatin C (7) showed dimer formation via the mechanism of three-dimensional domain swapping (8), and this observation has led to the suggestion that an analogous three-dimensional domain swapping process, but propagated in an open-ended fashion, could be the basis of the formation of cystatin C amyloid fibrils (7, 9). Dimerization via three-dimensional domain swapping has also been demonstrated in another amyloidogenic protein, the human prion protein (10), and recently, in a series of ingenious experiments, Lee and Eisenberg (11) have given indirect evidence that exchange of structural domains may, indeed, be involved in the aggregation of the prion protein into fibrillar form.

The general fold of monomeric inhibitors of the cystatin superfamily has been defined by the crystal structure of chicken cystatin (12). It consists of a long α-helix running across a large five-stranded antiparallel β-sheet (β1-β5). Native cystatin C contains four disulfide-paired cysteine residues, all in the C-terminal part of its sequence (Cys-73–Cys-83, Cys-
97–Cys-117). The symmetric dimer of wt cystatin C retains the secondary structure elements of the monomeric form but exchanges them between the two monomer-mimicking subunits. The domain that undergoes swapping consists of the α-helix and its two flanking β-strands (β1 and β2). It is connected to the C-terminal part of the protein (strands β3-β5) by a hinge region that in the monomeric molecule forms a β-hairpin loop. The native cysteine bridges, both located in the C-terminal part of the protein, do not interfere with the domain swapping process, in which the N- and C-terminal parts are exchanged. Although the crystal structure of L68Q cystatin C has not been determined yet, it is most likely very similar to that of wt cystatin C (13). The crystallographic model of the wt cystatin C dimer explains why the mutated protein is less stable and undergoes domain swapping dimerization much more easily (7). Because the altered residue is located in the central strand (β3) of the β-sheet and is covered by the α-helix, where it occupies a hydrophobic pocket formed by residues Val-31 and Tyr-34, the longer and hydrophilic glutamine side chain causes destabilization of the molecular α/β interface, leading to structural rearrangements and increased probability of partial unfolding. In the unfolded state, the newly exposed Gln-68 residue has favorable (as opposed to Leu-68) solvent contacts, thus further contributing to the loss of stability.

Considering the proposed model of fibrillogenesis by propagated three-dimensional domain swapping, it seems possible that inhibition of the domain swapping process should also suppress the entire process of dimerization and fibrillogenesis. The objective of the present work has been to produce two variants of both wt and L68Q cystatin C with disulfide bridges at positions selected to inhibit domain swapping without affecting the biological function of the four cystatin C variants and to investigate whether this would result in suppression of dimerization and fibril formation. In additional experiments investigating whether exogenous agents could also inhibit the formation of dimers of wt and L68Q cystatin C, a monoclonal antibody and carboxymethylpapain were used as templates to introduce the third point mutation L68Q, introducing the mutations F29C and M110C by the oligonucleotides L47C/L68Q/G69C (5'-TCAGGCTCAACGGCCGGC-3' and L68Q/G69C (5'-TTCTTTGAGCTTGGCCAGGG-3'), with a unique PstI site removed. Thereafter, the two different plasmids constructed (L47C/G69C and F29C/M110C) were used as templates to introduce the third point mutation L68Q, with an AluI site removed, using the primers L68Q (5'-TTCTTTGAGCTTGGCCAGGG-3') and L68Q/G69C (5'-TTCTTTGAGCTTGGCCAGGG-3'), with a unique PstI site recognition site and M110C (5'-TTGGCCAGGGCCACGCTTGTCGAAATCCACC-3'), with the unique PstI site lost. Thereafter, the two different plasmids constructed (L47C/G69C and F29C/M110C) were used as templates to introduce the third point mutation L68Q, with an AluI site removed, using the primers L68Q (5'-TTCTTTGAGCTTGGCCAGGG-3') and L68Q/G69C (5'-TTCTTTGAGCTTGGCCAGGG-3'), with a unique PstI site recognition site and M110C (5'-TTGGCCAGGGCCACGCTTGTCGAAATCCACC-3'), with the unique PstI site lost. Thereafter, the two different plasmids constructed (L47C/G69C and F29C/M110C) were used as templates to introduce the third point mutation L68Q, with an AluI site removed, using the primers L68Q (5'-TTCTTTGAGCTTGGCCAGGG-3') and L68Q/G69C (5'-TTCTTTGAGCTTGGCCAGGG-3'), with a unique PstI site recognition site and M110C (5'-TTGGCCAGGGCCACGCTTGTCGAAATCCACC-3'), with the unique PstI site lost. Thereafter, the two different plasmids constructed (L47C/G69C and F29C/M110C) were used as templates to introduce the third point mutation L68Q, with an AluI site removed, using the primers L68Q (5'-TTCTTTGAGCTTGGCCAGGG-3') and L68Q/G69C (5'-TTCTTTGAGCTTGGCCAGGG-3'), with a unique PstI site recognition site and M110C (5'-TTGGCCAGGGCCACGCTTGTCGAAATCCACC-3'), with the unique PstI site lost. Thereafter, the two different plasmids constructed (L47C/G69C and F29C/M110C) were used as templates to introduce the third point mutation L68Q, with an AluI site removed, using the primers L68Q (5'-TTCTTTGAGCTTGGCCAGGG-3') and L68Q/G69C (5'-TTCTTTGAGCTTGGCCAGGG-3'), with a unique PstI site recognition site and M110C (5'-TTGGCCAGGGCCACGCTTGTCGAAATCCACC-3'), with the unique PstI site lost. Thereafter, the two different plasmids constructed (L47C/G69C and F29C/M110C) were used as templates to introduce the third point mutation L68Q, with an AluI site removed, using the primers L68Q (5'-TTCTTTGAGCTTGGCCAGGG-3') and L68Q/G69C (5'-TTCTTTGAGCTTGGCCAGGG-3'), with a unique PstI site recognition site and M110C (5'-TTGGCCAGGGCCACGCTTGTCGAAATCCACC-3'), with the unique PstI site lost. Thereafter, the two different plasmids constructed (L47C/G69C and F29C/M110C) were used as templates to introduce the third point mutation L68Q, with an AluI site removed, using the primers L68Q (5'-TTCTTTGAGCTTGGCCAGGG-3') and L68Q/G69C (5'-TTCTTTGAGCTTGGCCAGGG-3'), with a unique PstI site recognition site and M110C (5'-TTGGCCAGGGCCACGCTTGTCGAAATCCACC-3'), with the unique PstI site lost.
purification and buffer exchange by gel chromatography on a Superdex HR 75 column as described above. The solutions of L68Q cystatin C and L68Q cystatin C stab1 were finally concentrated to ~0.2 mg/ml by ultrafiltration, whereas the final concentration of L68Q cystatin C stab2 was approximately 0.05 mg/ml. The L68Q cystatin C variants were stored at 4°C until used. The protein concentrations of the solutions of the cystatin C variants were determined by UV absorption spectrophotometry, using a molar extinction coefficient of ε = 11,100 M⁻¹·cm⁻¹ (A280, O.D. = 0.83) (22).

The isolated cystatin C variants were characterized by agarose gel electrophoresis at pH 8.6 (23) and with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (24) using precast NuPage gradient gels (4–12%) and the morpholineethanesulfonic buffer system at pH 6.4 described by the manufacturer. To verify the identity of protein bands after agarose gel electrophoresis, immunoblot analysis (25) with polyclonal antibodies against wt cystatin C was performed.

**Analysis of Disulfide Bridge Topology**—The positions of the disulfide bridges in wt cystatin C, wt cystatin C stab1, wt cystatin C stab2, and L68Q cystatin C were determined by mass spectrometry. The instrument was a matrix-assisted laser desorption time-of-flight mass spectrometer (Reflex III, Bruker Daltonics, Bremen, Germany). The samples (1–2 μg) were digested with 40 ng of sequencing grade trypsin (Promega, Madison, WI) in 25 μl ammonium bicarbonate at pH 7.8 for 12 h. The digestion buffer also contained 2 μM N-ethylmaleimide (MP Biomedicals, Irvine, CA) to avoid reshuffling of the cysteine bridges. After acidification with trifluoroacetic acid, a short purification step was made on C₁₈ ZipTips™ (Millipore, Bedford, MA) before drying the eluate. The apo cystatin C complex was deposited on the Anchorchip™ (Bruker Daltonics, Bremen, Germany) target plate before sample was applied. The analysis was performed in the reflector mode, averaging 50–100 individual laser shots.

**Determination of Equilibrium Constants for Dissociation (Kᵢ)**—**Of Complexes between Cystatin C Variants and Cathepsin B**—Methods used for active site titration and determination of equilibrium constants for dissociation (Kᵢ) of complexes between cystatin C and cysteine peptidases have been reviewed in detail (26). Briefly, the active concentrations of the different cystatin C variants, except L68Q cystatin C stab2, were determined by titration with papain and using benzoyl-DL-arginine 4-nitroanilide as the substrate. The papain used had previously been active site–treated using 1:3-carboxy-2,3-trans-epoxypropionyl-leucylamido-4- guanidino-butane. The assay buffer was 100 mM sodium phosphate, pH 6.5, containing 1 mM dithiothreitol and 2 mM EDTA to activate the enzyme. The absorbance of the cleaved 4-nitroanilide was measured at 405 nm in a Labsystems Multiskan Plus spectrophotometer.

Because of the low expression level of L68Q cystatin C stab2, titration of this protein was performed in a more dilute system than the one described above, using ~30 nM papain and the fluorogenic substrate Z-Phe-Arg-NHMec. This dilution does not influence the binding between the enzyme and its inhibitor because the dissociation constant of the papain-cystatin C complex is known to be in the femtomolar range (22). Two and a half μl of active site–treated papain solution was thus mixed with 5 μl of assay buffer and incubated for 15 min at 37°C. After addition of 2.5 μl of a solution of L68Q cystatin C stab2 in different dilutions, the mixtures were incubated for 30 min at 37°C. The mixture was then diluted 1:10 with 0.1% (v/v) Brij-35, and 2.5 μl of the diluted sample was added to 62.5 μl of 0.1% Brij-35, 25 μl of 100 mM sodium phosphate, pH 6.5, and 10 μl of the assay buffer solution producing a final Z-Phe-Arg-NHMec concentration of 20 μM. The samples were incubated at 37°C for 10 min, after which the reaction was stopped by adding 100 μl of 100 mM monochloracetic acid, pH 4.3. Fluorescence was measured in a FLUOstar OPTIMA instrument (BMG Labtechnologies, Offenburg, Germany) at excitation and emission wavelengths of 390 and 460 nm, respectively.

A similar assay (27) was used to measure the equilibrium constants for dissociation (Kᵢ) of complexes between cathepsin B and the different cystatin C variants. The substrate used was Z-Phe-Arg-NHMec (10 μM), and the assay buffer was 100 mM sodium phosphate, pH 6.0, containing 1 mM dithiothreitol and 2 mM EDTA. Steady-state velocities before (vₒ) and after (vᵢ) addition of inhibitor (I) were measured, and the apparent Kᵢ values were calculated as the slope from plots of [I][I/(1 – vᵢ/vₒ)] versus [I]/vₒ (28). A PerkinElmer LS-50 fluorimeter and excitation and emission wavelengths of 360 and 460 nm, respectively, were used for all cystatin C variants, except for L68Q cystatin C stab2. L68Q cystatin C stab2 was analyzed in the same way but at a final volume of 150 μl instead of 3 ml and with a FLUOstar OPTIMA fluorimeter with excitation and emission wavelengths of 390 and 480 nm, respectively.

**Dimerization Experiments**—A suitable in vitro system to promote dimerization of wt cystatin C was developed to allow investigations of the potential for dimer formation of wt cystatin C and ways to inhibit it. Guanidinium chloride was added to a solution of wt cystatin C at 0.5 mg/ml in PBS, to a final concentration of 0.5 M. The solution was then incubated at 37°C for up to 25 days. The two stabilized variants of wt cystatin C (wt cystatin C stab1 and wt cystatin C stab2) were incubated at the same conditions. L68Q cystatin C dimerizes spontaneously in physiological buffers, and its dimerization rate at 0.2 mg/ml was therefore studied in PBS at 37°C for up to 7 days. The dimerization rate of the stabilized L68Q cystatin C stab1 was studied at the same conditions. Because of low expression levels, L68Q cystatin C stab2 was tested at a lower concentration (~0.05 mg/ml). Dimers of both wt cystatin C and L68Q cystatin C display mobilities different from those of the monomeric proteins in agarose gel electrophoresis at pH 8.6 (4). Agarose gel electrophoresis was therefore used to follow the production of cystatin C dimers. Quantitative determinations were based upon densitometric scanning of agarose gel electrophorograms using an Epson Expression 1680 Pro scanner and the “Quantity One” program from Bio-Rad. Several samples were also analyzed by gel chromatography on a calibrated Superdex HR75 column to verify the results obtained by scanning of agarose gel electrophorograms.

Scanning of agarose gel electrophorograms could also be used to follow the production of cystatin C dimers in incubation mixtures containing carboxymethylmethylparain or monoclonal antibodies against cystatin C, because the complexes formed between these substances and cystatin C or L68Q cystatin C differed in mobility from those of monomeric and dimeric wt and L68Q cystatin C. The production, properties, and isolation of the IgG2b(c) murine antibody against cystatin C used in these experiments have been described previously (21). So have the production, properties, and isolation of the IgG2b(c) murine antibody against protein HC used in control experiments (29). The concentration of anticystatin C antibody was determined by UV absorption spectroscopy at 280 nm using a molar extinction coefficient of ε = 214,500 M⁻¹·cm⁻¹ (A₂₈₀, O.D. = 1.43). Papain (lyophilized powder, crystallized) obtained from Sigma was further purified by affinity chromatography at 4°C using a column of H-Gly-Gly-Tyr-Arg-Sepharose as described by Blumberg et al. (30) and used for the preparation of carboxymethylparain. To 1 ml of a solution of papain (7 mg/ml) in 0.2 M sodium phosphate buffer, pH 6.0, containing 4 mM cysteine and 2 mM EDTA, was added 0.1 ml of a solution of 250 mM iodoacetamide in 0.5 M sodium phosphate buffer, pH 6.0. After 15 min in room temperature, the mixture was dialyzed against PBS. The concentration of carboxymethylparain was determined by UV absorption spectroscopy at 280 nm using a molar extinction coefficient of ε = 58,500 M⁻¹·cm⁻¹ (A₂₈₀, O.D. = 2.39) (31).

**Amyloid Fibril Formation**—Wild type cystatin C, wt cystatin C stab1, and wt cystatin C stab2 were incubated at 37°C with 0.3 mg/ml in 10 mM glycine buffer, pH 2.0, containing 0.2% (w/v) sodium azide, at 48°C under constant stirring using a magnetic spinbar. The appearance of amyloid fibrils in the incubation mixtures was repeatedly analyzed for 4 weeks by electron microscopy and by the thioflavin T fluorescence assay (32, 33).

**Electron Microscopy of Amyloid Fibrils**—Protein samples (5 μl) 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 dried. The samples were then negatively stained with 2% (w/v) aqueous uranyl acetate and examined with a Philips CM10 electron microscope (Philips, Eindhoven, The Netherlands), operated at an excitation voltage of 80 kV.

**Quantitative Estimation of Amyloid Fibrils**—The formation of amyloid fibrils was estimated by the thioflavin T fluorescence assay as described by LeVine (32, 33). Samples of wt cystatin C, wt cystatin C stab1, and wt cystatin C stab2 (3 mg/ml) were diluted with 50 mM glycine-sodium hydroxide buffer, pH 8.5, containing 100 μM thioflavin T to calculate protein concentrations (mg/ml) (electrophoretic species). Measurements were carried out in polyurethane 96-well microtiter plates (Black Cliniplates, Thermo Labsystems, Helsinki, Finland) pretreated with Sigmacote (Sigma). The fluorescence was recorded using a FLUOstar OPTIMA fluorimeter at excitation and emission wavelength of 450 and 490 nm, respectively.

**RESULTS**

The conformational flexibility of monomeric wt and L68Q cystatin C allows these molecules to form dimers and possibly...
Abortion of Cystatin C Domain Swapping Prevents Dimerization

Fig. 1. A view of one half of the three-dimensional domain-swapped dimer of wt cystatin C, illustrating the positions selected in the present work for mutation to cysteine residues. The mutations L47C/G69C were designed to introduce a new disulfide bond linking the strands β2 and β3 of the β-sheet. The mutations F29C/M110C were designed to introduce a connection between the α-helix and strand β5. In the domain-swapped dimer of wt cystatin C, strand β2 and the α-helix are contributed by one of the monomers and strands β3 and β5 by the other. The figure, prepared using MOLMOL (16), is based on the coordinates deposited in the Protein Data Bank (accession code 1G96). The coordinates of the new cysteine residues (shown as ball-and-stick models) were obtained by mutating the coordinates of the original residues. The conformation of the side chains of the mutated cysteine residues could be easily adjusted manually in the molecular graphics program O (17) to achieve S-S distances in the paired cysteine residues of approximately 2–3 Å, without compromising stereochemistry. This is emphasized in the figure by the covalent disulfide bonds.

amyloid fibrils by domain swapping. To investigate whether suppression of this flexibility would also suppress the ability of the molecules to form dimers and fibrils, an attempt was made to produce two variants of each protein stabilized by an extra disulfide bridge for each variant. It was considered important to insert the new disulfide bridges in a way that would leave the general fold of cystatin C as intact as possible, preserving, for example, the capacity of the proteins to inhibit cysteine proteases. This was done by strategic insertion of new disulfide bridges aiming at anchoring the domain swapping subunits to the backbone of the cystatin C molecules without disruption of the general fold. In efforts to create the first type of stabilized monomeric wt and L68Q cystatin C molecules (called stab1), the mutations were selected to introduce a disulfide bridge between the β2- and β3-strands, which separate when dimeric cystatin C is formed (Fig. 1) (7). To create the second type of stabilized monomeric wt and L68Q cystatin C molecules (called stab2), the mutations were selected to introduce a disulfide bridge between the α-helix and the β5-strand, which also separate during the process of three-dimensional domain swapping of cystatin C (7).

Expression and Purification of the Stabilized Cystatin C Variants—An expression vector carrying the gene for wt cystatin C (14) was subjected to oligonucleotide-directed mutagenesis to create vectors with genes encoding L47C/G69C and F29C/M110C cystatin C as well as L47C/L68Q/G69C and F29C/L68Q/M110C cystatin C. After sequencing, the expression vectors were introduced into E. coli MC1061. It was thus possible to express two potentially stabilized variants of wt cystatin C (wt cystatin C stab1 and wt cystatin C stab2), and two potentially stabilized variants of L68Q cystatin C (L68Q cystatin C stab1 and L68Q cystatin C stab2). The expression levels of the cystatin C variants differed considerably, requiring different isolation procedures. Wild type cystatin C and its two variants were isolated by a two-step procedure consisting of anion exchange chromatography at pH 9.0, followed by gel chromatography (14, 20). Both wt cystatin C stab1 and wt cystatin C stab2 eluted from a calibrated Superdex HR 75 column at the position corresponding to monomeric wt cystatin C. The expression levels for the two purified protein variants were comparable with that of wt cystatin C with a yield of 3–15 mg of protein/liter of culture.

The expression levels of L68Q cystatin C, L68Q cystatin C stab1, and L68Q cystatin C stab2 were considerably lower (0.05–0.5 mg of protein/liter of culture) and these proteins could not be isolated using ion exchange chromatography. Concentrated periplasmic extracts of L68Q cystatin C and its two variants were therefore subjected to immunosorption as previously described (4), using a monoclonal anticystatin C antibody coupled to Sepharose beads. The proteins were eluted with a buffer containing 6 M guanidinium chloride. After concentration of the eluate, gel filtration on a Superdex HR 75 column in PBS at room temperature was used as the final purification step. The elution positions of L68Q cystatin C and its stabilized variants coincided with that of monomeric wt cystatin C.

Physicochemical Characterization of the Cystatin C Variants—The wt and L68Q cystatin C variants were analyzed by agarose gel and SDS-PAGE electrophoresis. Agarose gel electrophoresis demonstrated that the charges of all four variants at pH 8.6 were identical and agreed with those of wt and L68Q cystatin C (Figs. 2 and 3). SDS-PAGE electrophoresis showed that the apparent sizes of all the variants also were identical and in accordance with those of wt and L68Q cystatin C.

To verify that the positions of the disulfide bridges in wt cystatin C, wt cystatin C stab1, wt cystatin C stab2, and L68Q cystatin C were as expected, unreduced samples were incubated with trypsin and the generated peptides were analyzed by mass spectrometry. The digestions were made in the presence of N-ethylmaleimide (an alkylating agent) to avoid refolding of the disulfide bridges upon digestion. No alkylation of cysteines was observed, indicating that the disulfides were kept intact during the digestion procedure. Fragments corresponding to the anticipated disulfide bridges between residues 73 and 83, 97 and 117, 47 and 69, and between residues 29 and 110 could be identified, whereas no fragments corresponding to other disulfide bridges were found (Table II). Digestion of wt cystatin C stab2 also produced a fragment comprising three

Fig. 2. Agarose gel electrophoresis of wt cystatin C, wt cystatin C stab1, and wt cystatin C stab2. Solutions of wt cystatin C, wt cystatin C stab1, and wt cystatin C stab2 in phosphate-buffered saline, pH 7.4, containing 0.5 M guanidinium chloride were incubated at 37 °C for 7 days and analyzed by agarose gel electrophoresis at pH 8.6. The electrophoretic mobilities of monomeric and dimeric cystatin C are indicated by arrows. The anode and the point of application are marked by a plus sign and an arrow, respectively. Human blood plasma is used as a reference.
The anode and the point of application are marked by a plus sign and an arrow, respectively. Human blood plasma is used as a reference.

Oligopeptides with two disulfide bridges between the oligopeptides, compatible with the expected arrangement of the disulfide bonds in this wt cystatin C variant. A few peptides with intact trypsin cleavage sites were also identified, but all were compatible with the expected arrangement of the disulfide bridges.

To further examine whether or not the introduced disulfide bridges had significantly altered the tertiary structure of the cystatin C variants, the cysteine protease-inhibiting capacities of the proteins were investigated. This was done by determination of the equilibrium constants for dissociation (K_i) of complexes between cathepsin B and the different cystatin C variants using a continuous-rate enzyme analysis. It was observed that all four stabilized cystatin C variants as well as wt and L68Q cystatin C displayed virtually identical equilibrium constants for inhibition of cathepsin B (Table III).

**Dimerization of the Cystatin C Variants**—The crystal structure of dimeric wt cystatin C demonstrates that the dimer is formed via three-dimensional swapping of domains between the two participating wt cystatin C monomers (7). To compare the domain swapping potentials of the stabilized cystatin C variants with those of the parent molecules, wt and L68Q cystatin C, the capability of all the proteins to form dimers was investigated.

Although wt cystatin C crystallizes in dimeric form from concentrated solutions, it is known to be quite stable as a monomer under physiological conditions (4). It was, therefore, necessary to use mildly denaturing conditions when its potential for dimer formation was studied. We have established an in vitro system for wt cystatin C in PBS with 0.5 M guanidinium chloride at 37 °C as a suitable system to facilitate dimerization. Aliquots were removed at timed intervals and subjected to agarose gel electrophoresis to study the formation of dimers, which have higher anodal electrophoretic mobility than the monomers (Fig. 2). Densitometric scanning of the electropherograms was used to determine the ratio between the monomers and dimers. Furthermore, to verify the results, several samples were studied by gel chromatography. When samples of wt cystatin C were incubated at 37 °C, significant amounts of dimers were seen within 3 days, whereas wt cystatin C stab1 and wt cystatin C stab2 did not produce any dimers at all when incubated at identical conditions (Fig. 2). Indeed, no dimers of the stabilized wt cystatin C variants could be observed even after a prolonged incubation period of 28 days (Fig. 4). No difference in their potential for dimer formation could be found between the two stabilized variants.

In contrast to wt cystatin C, L68Q cystatin C dimerizes spontaneously within a short period of time in physiological buffers (4). Freshly isolated L68Q cystatin C was therefore incubated in PBS at 37 °C, and a significant amount of dimers could already be observed after 2 h (Fig. 3). When the two stabilized variants of L68Q cystatin C (L68Q cystatin C stab1 and L68Q cystatin C stab2) were incubated at the same conditions, no dimers were produced even after incubation periods of up to 7 days (Fig. 4). L68Q cystatin C stab1 and its L68Q cystatin C control were incubated at a concentration of 0.2 mg/ml. Because of the very low expression level of L68Q cystatin C stab2, this protein had to be tested in a lower concentration (0.05 mg/ml). L68Q cystatin C stab2 and its L68Q cystatin C control at the same concentration were therefore immunoprecipitated prior to staining for protein (Fig. 3).

**In Vitro Production of Amyloid Fibrils from wt Cystatin C and Its Stabilized Variants**—To investigate whether amyloid fibril production from wt cystatin C could be suppressed by stabilizing the monomeric form, an in vitro system was developed for production of fibrils from wt cystatin C. Several conditions for fibril production were examined, including different buffers (pH 2.0–7.8), different temperatures (37, 48, or 65 °C), different protein concentrations, and incubation with or without stirring. Fibrils of wt cystatin C were found to be produced in solutions of low pH, and the production was accelerated by high temperature and stirring. We selected a cystatin C concentration of 3 mg/ml, incubation in 10 mM glycine buffer, pH 2.0, and continuous magnetic stirring at 48 °C for fibril production in this study. Identical conditions were used to investigate fibril formation of wt cystatin C stab1 and wt cystatin C stab2. The fibrils showed tinctorial properties characteristic of amyloid when stained with both Congo red and thioflavin T, and the spectral alterations on binding of both dyes were typical for amyloid fibrils. Electron micrographs of the fibrils showed long, nonbranching, twisted, wirelike structures approximately 10 nm wide (Fig. 5). No fibrils could be detected in the solutions at the start of the incubation period. After approximately 1 day, a low amount of fibrils could be observed in the solution of wt cystatin C but not in the solutions of its two stabilized variants. After 7 days of incubation, a substantial amount of fibrils could be observed in the solution of wt cystatin C, whereas only low amounts of fibrils were present in the solutions of wt cystatin C stab1 and wt cystatin C stab2. A thioflavin T assay was used to quantitatively follow the production of fibrils from the three proteins. As can be seen in Fig. 6, wt cystatin C had produced a considerable amount of fibrils after 7 days of incubation and the amount of fibrils increased continuously for at least 3 more weeks, although at a lower rate than during the first week. In contrast, after incubation periods of more than 7 days, the amounts of fibrils produced from wt cystatin C stab1 or wt cystatin C stab2 corresponded to only approximately 20% of that produced from wt cystatin C (Fig. 6). The low expression levels of L68Q cystatin C and its variants did not allow studies of their capacities to form fibrils.

**Suppression of Dimerization of wt Cystatin C and L68Q Cystatin C by Monoclonal Antibodies and Carboxymethylpapain**—Although additional disulfide bridges introduced into wt and L68Q cystatin C seem to be able to suppress the capacity of these proteins to dimerize and produce amyloid fibrils, it is interesting from an inter alia therapeutic point of view to investigate whether exogenous agents might also display similar effects. We therefore investigated whether a monoclonal antibody raised against wt cystatin C (21) would be able to inhibit...
TABLE II
Tryptic digestion of wt cystatin C, wt cystatin C stab1, wt cystatin C stab2, and L68Q cystatin C analyzed by matrix-assisted laser desorption time-of-flight mass spectrometry

Cysteine residues participating in disulfide bridges are underlined. The number of intact trypsin cleavage sites in the peptides is shown in parentheses. The peptide specific for L68Q cystatin C is shown in italics.

<table>
<thead>
<tr>
<th>Cystatin C variant</th>
<th>Peptide sequences</th>
<th>Residues</th>
<th>Mass obtained</th>
<th>Mass calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt cystatin C</td>
<td>TTCTK + TQPNLDNCPFHDQPHLK</td>
<td>71–75 (0)</td>
<td>2554.3</td>
<td>2554.2</td>
</tr>
<tr>
<td>wt cystatin C</td>
<td>TTCTKTPNLNCDFPDHQPHLK</td>
<td>76–92 (0)</td>
<td>2536.3</td>
<td>2536.2</td>
</tr>
<tr>
<td>wt cystatin C stab1</td>
<td>ACFSQYAVPQUQMTLSTK + STCQDA</td>
<td>95–114 (0)</td>
<td>2899.4</td>
<td>2899.3</td>
</tr>
<tr>
<td>wt cystatin C stab2</td>
<td>ACQVVR + QIVAGVNYFDVLQG</td>
<td>46–51 (0)</td>
<td>2511.2</td>
<td>2511.3</td>
</tr>
<tr>
<td>wt cystatin C stab1</td>
<td>TQPNLDNCPFHDQPHLK</td>
<td>71–75 (0)</td>
<td>2554.3</td>
<td>2554.2</td>
</tr>
<tr>
<td>wt cystatin C stab2</td>
<td>ACFSQYAVPQUQMTLSTK + STCQDA</td>
<td>95–114 (0)</td>
<td>2899.4</td>
<td>2899.3</td>
</tr>
<tr>
<td>L68Q cystatin C</td>
<td>TTCTK + TQPNLDNCPFHDQPHLK</td>
<td>71–75 (0)</td>
<td>2554.3</td>
<td>2554.2</td>
</tr>
<tr>
<td>L68Q cystatin C stab1</td>
<td>ACFSQYAVPQUQMTLSTK + STCQDA</td>
<td>95–114 (0)</td>
<td>2899.4</td>
<td>2899.3</td>
</tr>
<tr>
<td>L68Q cystatin C stab2</td>
<td>ACFSQYAVPQUQMTLSTK + STCQDA</td>
<td>95–114 (0)</td>
<td>2899.4</td>
<td>2899.3</td>
</tr>
</tbody>
</table>

Table III
Equilibrium constants for dissociation ($K_i$) of the complexes between cathepsin B and the different cystatin C variants

The standard deviation (S.D.) and number of measurements (n) used to calculate the mean $K_i$ values given are indicated.

<table>
<thead>
<tr>
<th>Cystatin C variant</th>
<th>$K_i$</th>
<th>S.D.</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt cystatin C</td>
<td>0.37</td>
<td>±0.068</td>
<td>6</td>
</tr>
<tr>
<td>wt cystatin C stab1</td>
<td>0.23</td>
<td>±0.022</td>
<td>5</td>
</tr>
<tr>
<td>wt cystatin C stab2</td>
<td>0.35</td>
<td>±0.012</td>
<td>5</td>
</tr>
<tr>
<td>L68Q cystatin C</td>
<td>0.40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L68Q cystatin C stab1</td>
<td>0.36</td>
<td>±0.049</td>
<td>5</td>
</tr>
<tr>
<td>L68Q cystatin C stab2</td>
<td>0.43</td>
<td>±0.032</td>
<td>6</td>
</tr>
</tbody>
</table>

* The $K_i$ of L68Q cystatin C has previously been determined by Abrahamson and Grubb (4).
form dimers by the process of three-dimensional domain swapping (7, 10). The concept of three-dimensional domain swapping, as defined by Eisenberg, refers to the situation where an element of the tertiary structure of a protein has been replaced with an identical piece from another protein molecule (8). In addition to being a mechanism for dimerization of proteins, propagated three-dimensional domain swapping has also been suggested as a mechanism for fibril formation of amyloidogenic proteins (7, 9–11, 39–42). This hypothesis has found confirmation in the recent experiments of Lee and Eisenberg (11), who not only provided evidence that the three-dimensional domain swapping reported for the prion protein (10) is indeed involved in amyloid fibril formation, but also demonstrated, for the first time, that such fibrils obtained from recombinant material carry a “seeding effect,” thus resembling the replication process of an infectious agent.

The general fold of monomeric proteins of the cystatin superfamily has been defined by the crystal structure of chicken cystatin and consists of a long β-helix running across a large β-sheet containing five antiparallel β-strands (β1–β5) (12). The crystal structure of dimeric wt cystatin C has revealed that the structural fragment that undergoes swapping consists of the β-helix and the flanking β1- and β2-strands (7). If this type of domain swapping indeed is the pathophysiological mechanism of dimerization and fibrillization of wt cystatin C and of the naturally occurring even more fibrillogenic variant, L68Q cystatin C, it should be possible to inhibit these processes by...
Abortion of Cystatin C Domain Swapping Prevents Dimerization

Fig. 7. Suppression of dimer formation of wt cystatin C and L68Q cystatin C by monoclonal antibodies (A) and carboxymethylpapain (B). Wild type cystatin C (●) (0.5 mg/ml) was incubated at 37 °C for 4 days in phosphate-buffered saline, pH 7.4, containing 0.5 M guanidinium chloride, with different amounts of a monoclonal antibody against cystatin C (A) or different amounts of carboxymethylpapain (B). L68Q cystatin C (○) (0.2 mg/ml) was incubated at 37 °C for 30 min in phosphate-buffered saline, pH 7.4, with different amounts of a monoclonal antibody against cystatin C (A) or different amounts of carboxymethylpapain (B). The degree of suppression of dimer formation was calculated from the amounts of dimers formed without additions of monoclonal antibodies or carboxymethylpapain to the solutions of wt cystatin C or L68Q cystatin C.

The capacity of the two variants of wt and L68Q cystatin C to form dimers was then investigated. Because L68Q cystatin C dimerizes spontaneously in physiological buffers, even at room temperature (4), and can be found in patients (5), the capacity of the two L68Q variants to dimerize was investigated using a physiological buffer and the temperature of 37 °C. Whereas a major part of L68Q cystatin C forms dimers at these conditions within 30 min, the two L68Q variants did not form any detectable amounts of dimers even after incubation periods of 7 days. This strongly indicates that the introduction of the disulfide bridges in the L68Q variants did stabilize the molecules in a way that prevents dimerization. In contrast to L68Q cystatin C, wt cystatin C forms dimers very slowly in physiological buffers, even at room temperature (4), and can be found in patients (5). The capacity of the two stabilized variants of wt and L68Q cystatin C to form dimers was then investigated. Because L68Q cystatin C dimerizes spontaneously in physiological buffers, even at room temperature (4), and can be found in patients (5), the capacity of the two L68Q variants to dimerize was investigated using a physiological buffer and the temperature of 37 °C. Whereas a major part of L68Q cystatin C forms dimers at these conditions within 30 min, the two L68Q variants did not form any detectable amounts of dimers even after incubation periods of 7 days. This strongly indicates that the introduction of the disulfide bridges in the L68Q variants did stabilize the molecules in a way that prevents dimerization. In contrast to L68Q cystatin C, wt cystatin C forms dimers very slowly in physiological buffers (4, 6). Therefore, to be able to investigate the stabilizing effect of the disulfide bridges introduced in the variants of wt cystatin C, a mildly denaturing system at pH 7.4 and 37 °C containing 0.5 M guanidinium chloride was used. In this system, approxi-
mately 50% of wt cystatin C formed dimers within 3 days, whereas the two stabilized variants of wt cystatin C did not form any detectable amounts of dimers even after prolonged incubation periods of 28 days. These results also indicate that the new disulfide bridges introduced in the wt cystatin C variants do prevent dimerization. The mechanisms for stabilization of the variants of both wt and L68Q cystatin C are probably identical, with the disulfide bridges preventing the partial unfolding that is a prerequisite of the domain swapping occurring during the formation of cystatin C dimers (7). Furthermore, both locations of the disulfide bridge, between the β2- and β3-strands or between the α-helix and the β5-strand, seem to be equally efficient for the stabilization of wt and L68Q cystatin C. The strategy to introduce extra disulfide bonds to increase the stability of engineered proteins has been used with success previously, e.g., for lysozyme and human RNase I (44, 45), but to the best of our knowledge the presently described use of new disulfide bridges to prevent domain swapping has not been reported before.

It has been suggested by several authors that three-dimensional domain swapping is involved not only in the formation of protein dimers but also in the formation of amyloid fibrils (7, 9–11, 39–42). Originally a mere speculation, this hypothesis has recently become a very real possibility, particularly in the light of the discoveries involving prion proteins and cystatin C (7, 9–11). It was therefore of interest to investigate whether prevention of domain swapping would have an inhibitory effect not only on dimer formation but also on the formation of amyloid fibrils. Although conditions producing fibrils of cystatin B have been described (46), we were not able to produce cystatin C amyloid fibrils using these conditions. We, therefore, tested several different conditions and were finally able to establish a system for the production of fibrils of wt cystatin C. These fibrils displayed the tinctorial properties described for amyloid fibrils when stained with Congo red or thioflavin T and showed the typical fibrous appearance when studied by electron microscopy. This system was used to compare the fibril forming capacity of wt cystatin C and its two variants stabilized by the engineered disulfide bridges. A quantitative method, based upon binding of thioflavin T, was used to follow the development of fibrils from the three cystatin C variants. Considerable amounts of fibrils were formed from wt cystatin C after incubation periods of 7 days or more, whereas the two stabilized forms of wt cystatin C only formed small amounts of fibrils, corresponding to no more than 20% of the fibrils formed from wt cystatin C. Prevention of domain swapping, therefore, seems to be able to substantially reduce the formation of amyloid fibrils, at least in the system tested.

It is not clear why preventing domain swapping completely inhibits dimerization of cystatin C, whereas it only inhibits approximately 80% of the ability of cystatin C to form amyloid fibrils. However, it should be remembered that the systems used to induce dimerization of the parent cystatin C molecules and the system used to form amyloid fibrils are very different. Indeed, the system used for production of amyloid fibrils involved strongly acidic conditions, and it has been suggested by Dobson that most, if not all, proteins will produce fibrils if sufficiently strong denaturing incubation conditions are used (47). It is thus possible that, in the harsh conditions used for induction of fibril formation, even the stabilized protein variants became unstable, i.e., partially unfolded, and entered on the aggregation pathway. Of course, there is still another possibility that the formation of fibrils could proceed in several ways. In such a scenario, prevention of cystatin C domain swapping in the presently used system for production of fibrils would completely abolish one important way of fibril formation but not necessarily other fibril forming pathways.

Although the data presented here indicate that prevention of domain swapping of cystatin C also will prevent, or substantially decrease, dimer and fibril formation, it should be remembered that the in vitro conditions used drastically differ from the tissue milieu in which amyloid fibrils are formed in amyloid disorders. It is also still an open question whether or not it is the fibrils per se or other smaller oligomers that contribute to the pathogenic symptoms of amyloid disorders (38, 48). It has been observed that, when different proteins are induced to form fibrils in vitro, oligomers that form early in the process are toxic to cells whereas the fibrils themselves are nontoxic (49, 50). It has also been stated that the pathophysiological roles of oligomers and fibrils must be clarified before efficient treatment modalities for amyloid disorders can be obtained (48). Although this might be true, the underlying mechanism for the formation of potentially toxic oligomeric precursors is presumably the same as that for fibril formation, which means that substances known to inhibit fibril formation also might inhibit the formation of their oligomeric precursors.

Although the production of cystatin C variants stabilized against domain swapping is very useful in demonstrating that prevention of domain swapping has the capacity to inhibit formation of dimers and amyloid 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. We therefore studied whether a monoclonal antibody raised against wt cystatin C could inhibit the cystatin C dimerization process and found that the antibody could suppress the dimerization of both wt and L68Q cystatin C. This observation might be related to the discovery that antibodies to surface epitopes of PrPSc can inhibit the generation of the amyloidogenic molecular species PrPSc and thereby interfere with prion biogenesis (51). It has been speculated that the effect of the antiprion antibodies was the result either of their stabilization of PrPSc, preventing its refolding into PrPSc, or of their inhibition of the interaction between PrPSc and PrPSc (51). The molar ratio between the antiprion antibodies and the prion proteins was not investigated, but the simple system used in the present investigation allowed the calculation of the effective antibody:cystatin C molar ratio. Surprisingly, this was found to be very low, and a molar ratio of 1:50 inhibited more than 50% of the dimerization of both wt and L68Q cystatin C in the systems tested. The reason that almost catalytic amounts of antibodies were effective in inhibiting dimerization is encouraging from a therapeutic point of view, but the molecular mechanism is not apparent. However, it has previously been observed that low molar amounts of a protein might inhibit fibrillogenesis of another protein. Specifically, gelsolin has been shown to inhibit in vitro fibrillization of synthetic Aβ1–40 and Aβ1–42 at a gelsolin to Aβ molar ratio of 1:40 (52). It has also been described that IgG2b antibodies against the N-terminal Aβ4–10 epitope have the capacity to promote Aβ fibril dissolution in vitro at antibody:Aβ ratios of 1:20–1:50 (53). It is conceivable that the effect of low molar amounts of the anticystatin C antibody might be caused by the preferential reaction of the antibody with those partially unfolded cystatin C molecular species that are directly involved in the dimer producing domain swapping process. Another mechanism might be that the antibody preferentially reacts with dimeric cystatin C and monomerizes it in the process. Further investigations are obviously required to elucidate the mechanism through which catalytic amounts of anticystatin C antibodies inhibit the formation of dimeric cystatin C.

It is also noteworthy that carboxymethylpapain, an active
site-alkylated inert derivative of the cysteine protease papain, can inhibit the dimerization of wt and L68Q cystatin C at low molar ratios of carboxymethylpapain to cystatin C. It is known that papain and carboxymethylpapain strongly bind to the inhibitory centers of monomeric wt and L68Q cystatin C (4, 54). Dimeric cystatin C, in which the domain swapping rearranges inhibitory centers of monomeric wt and L68Q cystatin C (4, 54).

Molar ratios of carboxymethylpapain to cystatin C. It is known that the cyclic peptide Aplidine inhibits aggregation of the prion peptide PrP in vitro. In addition, it has been observed that the cyclic peptide Aplidine inhibits aggregation of the prion peptide PrP at the Poznan Metropolitan Supercomputing and Networking Center.

Acknowledgment—Some of the calculations for this work were performed at the Poznan Metropolitan Supercomputing and Networking Center.

REFERENCES