Acta Biochimica Polonica

Vol. 56 No. 3/2009, 455–463

on-line at: www.actabp.pl

Regular paper

Governing the monomer-dimer ratio of human cystatin C by single amino acid substitution in the hinge region★

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Received: 14 May, 2009; revised: 02 July, 2009; accepted: 22 July, 2009
available on-line: 27 July, 2009

Three dimensional domain swapping is one of the mechanisms involved in formation of insoluble aggregates of some amyloidogenic proteins. It has been proposed that proteins able to swap domains may share some common structural elements like conformationally constrained flexible turns/loops. We studied the role of loop L1 in the dimerization of human cystatin C using mutational analysis. Introduction of turn-favoring residues such as Asp or Asn into the loop sequence (in position 57) leads to a significant reduction of the dimer fraction in comparison with the wild type protein. On the other hand, introduction of a proline residue in position 57 leads to efficient dimer formation. Our results confirm the important role of the loop L1 in the dimerization process of human cystatin C and show that this process can be to some extent governed by single amino acid substitution.

Keywords: human cystatin C, dimerization, hinge loop, conformational tension, point mutation

INTRODUCTION

Oligomerization of proteins *via* a mechanism involving mutual exchange of their fragments, also called three dimensional (3D) domain swapping, is an intriguing phenomenon. Named by Eisenberg nearly twenty years ago (Bennett *et al*., 1994), but known much earlier (Crestfield *et al*., 1962; London *et al*., 1974; Anderson *et al*., 1981), today it describes a feature shared by more than 60 proteins (structures deposited in the PDB database), many of them amyloidogenic (Bennett *et al*., 2006). Interestingly, these proteins do not display sequence or structural homologies, and the swapped domains can be located in the C- or N-terminal parts and significantly differ in size and structure (Liu & Eisenberg, 2002; Gronenborn, 2009). Prediction of the propensity of a particular protein to swap domains is therefore very difficult, however, there are a few hypotheses concerning identification of topological (Ding *et al*., 2006), amino acid sequence-related, or structural (Dehouck *et al*., 2003) determinants associated with such a propensity. The conformational changes that accompany domain swapping process are in many cases centered on a few amino-acid residues creating flexible loops and turns. The conformational tension arising from the distortion of dihedral angles ϕ and ψ of amino-acid residues in the turn regions (Dehouck *et al*., 2003) or from the presence of specific residues like proline (Bergdoll *et al*., 1996; Rousseau *et al*., 2003) may account for a lowered stability, forcing conformational changes that often lead to opening of the turn and, finally, to dimerization or oligomerization of the protein through 3D do-

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★Preliminary report of work reported in this publication was presented during 30th European Peptide Symposium, 2008, Helsinki, Finland (Szymańska A *et al*., 2009, The influence of human cystatin C hinge loop L1 on its dimerization and oligomerization propensities. *Proceedings of the 30th European Symposium*, ed. H. Lankinen, pp 630–631, Helsinki).

Abbreviations: hCC, human cystatin C; OmpA, outer membrane protein A; PBS, phosphate-buffered saline; SEC, size exclusion chromatography; 3D, three dimensional; wt, wild type.

main swapping. Such a turn destabilization effect is observed, e.g., in the cystatin family of cysteine protease inhibitors. Different members of this family have been shown to dimerize and oligomerize by the exchange of structural elements (Jaskólski, 2001; Staniforth *et al*., 2001; Janowski *et al*., 2001; 2005; Sanders *et al*., 2004; Nilsson *et al*., 2004; Jenko *et al*., 2007; Wahlbom *et al*., 2007).

Human cystatin C (hCC) belongs to the family II of the cystatin superfamily (Turk & Bode, 1991). This relatively small, basic, single chain protein (120 amino acids, 13.3 kDa) is a main regulator of cysteine proteases at physiological and pathological conditions (Henskens *et al*., 1996). The tertiary structure of hCC resembles that of other members of the cystatin family with a five-stranded anti-parallel β-sheet wrapped around a central α-helix (Bode *et al*., 1988; Martin *et al*., 1995) (Fig. 1a). At physiological conditions, hCC is a monomeric protein, but the crystal structure of the monomer has not been described yet, since under crystallization conditions cystatin C forms domain-swapped dimers (Fig. 1b) (Janowski *et al*., 2001; 2005). Dimerization of hCC can also be induced *in vitro* by incubation with a low concentration of a mild denaturant or at elevated temperature (Ekiel & Abrahamson, 1996).

In the dimerization process, two hCC molecules exchange N-terminal segments consisting of the α-helix, two β-strands and loop L1, which leads to the creation of a new, elongated interdomain interface (βL in Fig. 1b). Loop L1 of wild type hCC is the only part of the protein undergoing a significant change during dimerization as it transforms from a β-turn to an extended β-strand. It has been proposed (Staniforth *et al*., 2001) that this structural element may serve as a molecular spring facilitating dimerization. Experimental (Staniforth *et al*., 2001) and theoretical (Dehouck *et al*., 2003; Rodziewicz-Motowidło *et al*., 2009) studies have revealed that this region of the cystatin fold is conformationally unstable, mostly due to the valine residue located near the top of the loop L1 (Val57 for hCC). The values of the ψ and φ angles for this residue are not optimal (Rodziewicz-Motowidło *et al*., 2009), which therefore might contribute to the propensity of the protein to undergo domain swapping.

It should be mentioned that a naturally occurring point mutant (L68Q) of hCC forms dimers and subsequently higher oligomers more easily and at lower concentrations than the wild type protein (Abrahamson & Grubb, 1994; Nilsson *et al*., 2004). It is believed that mutation of the hydrophobic leucine residue to the hydrophilic glutamine locally destabilizes hydrophobic interactions and leads to fast dimer formation (Janowski *et al*., 2001; Rodziewicz-Motowidło *et al*., 2006). This effect is absent in the wild type hCC and therefore can not explain its dimerization propensity which, albeit lower than that of hCC L68Q, is still significant. Such observation leads to the conclusion that mutation in position 68 may be a trigger which facilitates the dimerization process and other structural features, common for wt hCC and the L68Q mutant, such as turn-formation propensity and its stability must contribute to their ability to swap domains.

In our recent theoretical studies (Rodziewicz-Motowidło *et al*., 2009) we have reported that position 57 in hCC could be crucial for dimer formation. The aim of the present study was to obtain hCC mutants with different substitutions in position 57 and to check their dimer formation propensity. We decided to produce two mutants of hCC in which Val57 residue was substituted by asparagine or aspartic acid residues which, according to the results of our theoretical studies (Rodziewicz-Motowidło *et al*., 2009), as well as the know propensity of Asp and Asn to stabilize β-turns (Wilmot & Thornton, 1988) should enhance monomer stability and inhibit dimer

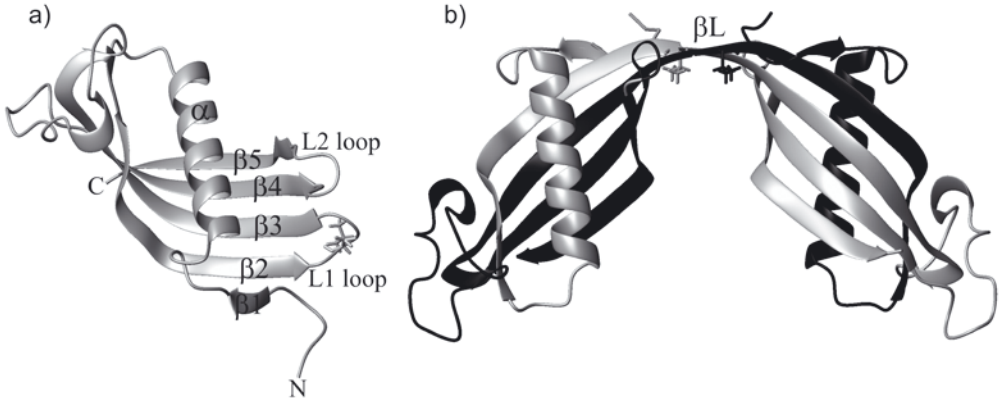

Figure 1. Models of human cystatin C monomer and dimer.
a) Monomer of hCC with selected β-strands, α-helix and L1, L2 loops constructed using X-ray crystal structure of dimeric wt hCC as described by Rodziewicz-Motowidło *et al*., 2006); **b)** Dimeric structure of hCC determined by crystallization (PDB code 1R4C). The domain swapped monomers are presented in gray and dark gray, and valine 57 is shown as sticks. Figure was prepared using MOLMOL program (Koradi *et al*., 1996).

formation. Additionally, since wild type hCC does not contain a proline residue in the hinge loop but is capable of undergoing 3D domain swapping, we also decided to obtain a proline mutant in position 57. Studies on the dimerization propensities of this hCC variant can deliver further evidence for verification of the "proline hinge" hypothesis (Bergdoll *et al*., 1996).

MATERIALS AND METHODS

General. Oligonucleotides for site directed mutagenesis and dNTPs were purchased from Oligo.pl (Warszawa, Poland). DNA sequencing was performed in the Laboratory for Nucleic Acid and Protein Detection (Pomeranian Science and Technology Park, Gdynia, Poland). *Pfu* polymerase was purchased from Fermentas. S-Sepharose matrix, Superdex 75 PC 10/300, Superdex 75 PC 3.2/30 columns and Gel Filtration Low Molecular Weight Calibration Kit were from GE Healthcare (previously Amersham Biosciences). DNA purification kit was from A&A Biotechnology (Gdańsk, Poland). CD spectra were obtained using a J-815 polarimeter (Jasco). Mass spectra were obtained using a BIFLEX III MALDI-TOF spectrometer (Bruker) or an LCMS-ESI-IT-TOF Liquid Chromatograph Mass Spectrometer (Shimadzu). Unless specified otherwise, all used reagents were of molecular biology or analytical grade.

Bacterial strains, plasmids and mutagenesis. Plasmid pHD313 encoding human cystatin C gene as a fusion protein with OmpA signal peptide for periplasmic expression (Abrahamson *et al*., 1988) was used for construction of new hCC variants. Point mutations were introduced by means of oligonucleotide-directed mutagenesis using *Pfu* polymerase according to the protocol provided by the manufacturer. DNA was amplified in *Escherichia coli* DH5α competent cells and purified using DNA purification kit. All mutations were verified by DNA sequencing.

Protein expression and purification. Protein were produced in *E. coli* C41(DE3) strain. Protein expression was induced when the optical density (OD_{600}) of the culture reached about 0.7 by quickly rising the temperature to 42°C. The temperature was next lowered to 40°C and the growth was continued for 3 h. The harvested bacteria were re-suspended in a buffer containing 20 mM Tris, pH 7.5, 10% glycerol (20 ml/l culture) and flash-frozen. Next the sample was thawed on ice and the freeze/thaw cycle was repeated. This treatment leads to partial breakage of bacterial outer membrane and release of proteins from the periplasmic space. Cells were then subjected to cold osmotic shock (Neu & Heppel, 1965) and appropriate fractions were collected. Proteins were purified by ion-exchange chromatography on S-Sepharose and eluted with a linearly increasing salt concentration (0 to 0.5 M NaCl in 20 mM Tris, 1 mM benzamidinium chloride, pH 7.5). Fractions containing pure hCC were collected, extensively dialyzed against 10 mM ammonium bicarbonate, pH 8.0, and lyophilized. When this procedure did not yield homogeneous samples, the lyophilized protein was dissolved in 20 mM ammonium bicarbonate, pH 8.0 and further purified on FPLC Superdex 75 PC 10/300 in the same buffer. Pure protein fractions were lyophilized and stored at –20°C. The purity of the proteins was confirmed using electrophoresis, gel filtration and mass spectrometry.

Circular dichroism measurements. CD spectra of wt hCC and its variants were measured in PBS (pH 7.4) in a 1 mm cell on a Jasco J-815 spectropolarimeter (Jasco Inc., Easton, Maryland, USA) at 20°C. Protein concentration was 0.1 mg/ml for far-UV CD and 1 mg/ml for near-UV CD. Three scans in the range 195–330 nm were collected and averaged. All spectra were corrected for the buffer signal.

Dimer formation. Dimerization experiments for all protein variants studied were performed in PBS containing 0.5 M or 1.0 M guanidine hydrochloride (Gdn·HCl) at 37°C with the protein at 0.5 mg/ml concentration. The progress of dimer formation was checked using gel filtration on Superdex 75 PC 3.2/30 and agarose gel electrophoresis. The column was calibrated using Gel Filtration Low Molecular Weight Calibration Kit containing Blue Dextran (2000 kDa, for column void volume determination), RNase A (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa) and albumin (67 kDa) (GE Healthcare).

Electrophoresis. For routine applications (monitoring protein expression, purification and purity) 15% SDS/PAGE in Tris/glycine Laemmli system was used. Gels were stained with Coomassie Brilliant Blue G-250. The progress of dimer formation was also monitored by native agarose gel electrophoresis on 1 mm, 1% agarose gels in 75 mM barbiturate buffer, pH 8.6. Gels were fixed in picric acid and stained with Coomassie Brilliant Blue R-250.

Gel filtration. Analytical gel filtration was performed on a Superdex 75 PC 3.2/30. Proteins were eluted with 150 mM ammonium bicarbonate, pH 7.4 at 0.1 ml/min flow rate.

RESULTS

Protein expression and purification

Wild type cystatin C (wt hCC) and its variants were expressed from pHD313 plasmid, in which

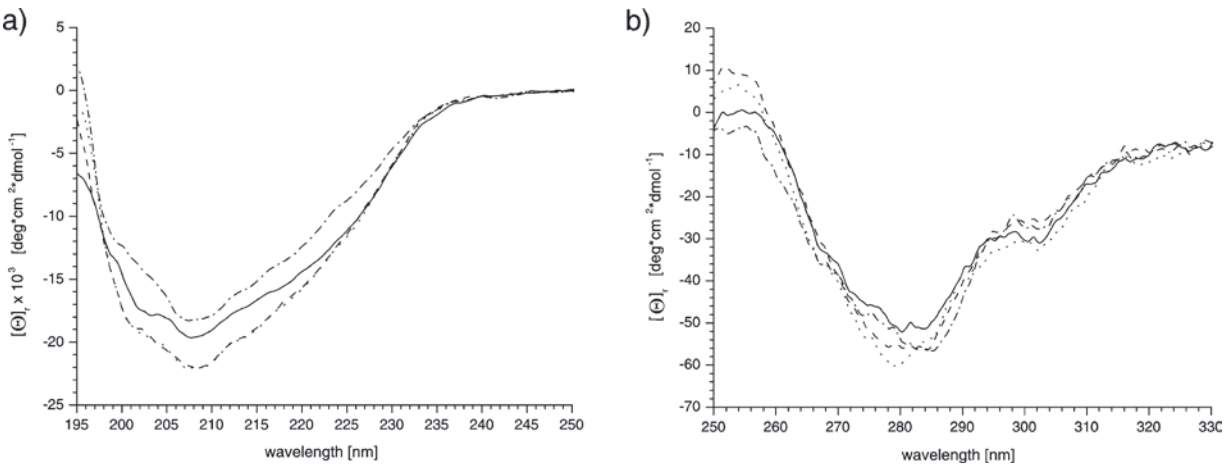

Figure 2. Circular dichroism spectra of wt hCC and its variants.
a) Far-UV CD and **b)** near-UV CD spectra of wt hCC (solid line) and its mutants: V57D (dash), V57N (dot) and V57P (dash dot) recorded in 1-mm cuvette in PBS at 20°C. The concentration of proteins was 0.1 mg/ml for the far-UV range and 1 mg/ml for near-UV.

protein expression is under the control of temperature-regulated repressor cI and P_R promoter from λ phage. Since the temperature sensitivity of the new proteins was not known, the expression conditions had to be optimized. According to our observations, the best results were obtained using a slightly lowered expression temperature (40°C) and induction at an OD_{600} below 1. Using this procedure we did not observe significant differences in the expression level of the mutants comparing to the wild type protein, and we were able to obtain up to 15 mg of the pure protein from 1 l of culture. The proteins were expressed as fusions with the signal peptide from OmpA (outer membrane protein A) and therefore were exported to the periplasmic space. During this process the signal peptide is cleaved by signal peptidase and full length cystatin C is obtained (Abrahamson *et al*., 1988). For the purification of recombinant proteins expressed to the periplasmic space cold osmotic shock is the most commonly used procedure. We used a similar approach, but during the analysis of the fractions we have observed significant leaking of proteins from the periplasmic space into the re-suspension buffer (20 mM Tris, pH 7.5, 10% glycerol) due to partial breakage of the cells during freezing. The amount of hCC in this fraction was high and comparable with the concentration in the periplasmic extract, but the purity was much higher. In order to further weaken the cells, bacteria in the re-suspension buffer were subjected to repeated freeze/thaw treatment and spun down. The supernatant was stored for further purification and the pellet was used for the cold osmotic shock. All fractions containing hCC were collected and purified using ion-exchange chromatography on S-Sepharose. In most cases this procedure yielded homogeneous proteins. If not, the proteins were subjected to gel filtration. Pure proteins were lyophilized and stored at –20°C. Mass spectra analysis confirmed that all hCC variants were correctly processed resulting in full length proteins with the planned mutation.

Physicochemical characterization of hCC variants

In order to check the influence of the mutations in the loop L1 on the structure of hCC, circular dichroism spectra of all variants were measured in PBS at 20°C. For all hCC variants CD spectra characteristic for a protein containing α-helical, β-strand, turns and random-coil structural elements were observed (Fig. 2a). The near-UV CD spectra (Fig. 2b) of wt hCC and the V57D mutant are similar, indicating that the introduced mutation has minor effect on the tertiary structure. For the V57N mutant a deeper minimum at 280 nm is observed than for the other proteins, whereas the minimum of hCC V57P variant is shifted to 285 nm. These differences may be due to different oligomeric state or differences in the compactness of the protein.

The stability of the proteins was evaluated using thermal denaturation monitored by measurement of ellipticity at 218 and 222 nm[1]. For all the mutants cooperative, two-state unfolding was observed at both wavelengths as opposed to the wild type protein which showed more complex behavior. The V57P mutant was the only one with a decreased

[1]Jankowska E, Orlikowska M, Radulska A, Szymańska A (2009) Are V57 mutants of amyloidogenic protein — human cystatin C more or less resistant to denaturation conditions? *Proceedings of the 30th European Symposium*, ed. H. Lankinen, pp 583–584, Helsinki.

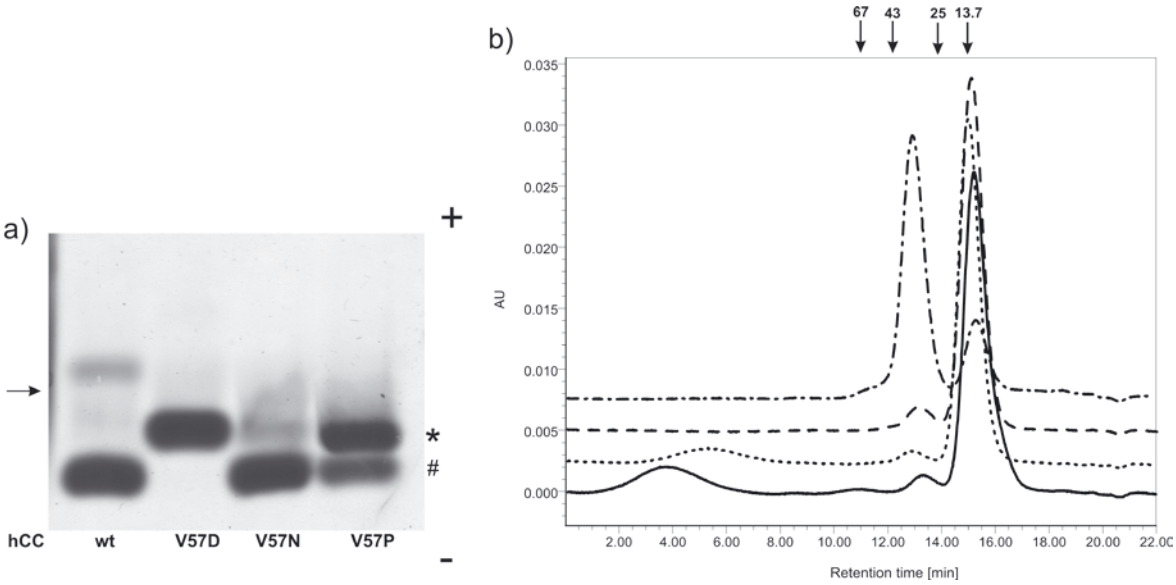

Figure 3. Native agarose electrophoresis and gel filtration of wt hCC and its variants.
a) Agarose gel electrophoresis of 1 mg/ml solutions of the studied proteins in PBS. Sample application position is marked with an arrow, the positions of bands corresponding to monomeric (#) and dimeric (*) hCC are also shown. **b)** Gel filtration chromatograms of the same samples. The elution position of molecular mass standards (in kDa) are marked with arrows.

thermal stability, with the temperature of half-denaturation t_m = 71°C, which is lower by 10°C than that for the other proteins. This result suggests that this particular amino acid substitution has the biggest impact on the stability of hCC and also, most likely, on its properties.

The electrophoretic mobility of all the studied proteins in an agarose gel was also checked. This technique had earlier been shown to allow simultaneous observation of different oligomeric forms of cystatin C based on differences in the mobility of protein dimers and monomers (Ekiel & Abrahamson, 1996; Stachowiak *et al.*, 2004). As expected, substitution of the neutral valine residue by a polar but not charged asparagine did not change the position of the protein band comparing to wild type cystatin C (Fig. 3a). The V57D mutant, bearing at pH 8.6 (electrophoresis conditions) the net charge lower by one comparing to wt hCC showed a slightly smaller shift towards the cathode, comparable with the one observed for the dimeric form of cystatin C. The proline mutant showed two bands: a major one with an electrophoretic mobility expected for the dimer and a minor one expected for the monomeric protein. In order to verify the above results gel filtration was performed (Fig. 3b). The retention times of the aspartic acid and asparagine mutants were similar to that of the wild type protein (R_T = 14.994 min for V57D; R_T = 15.105 for V57N, and R_T = 15.211 min for wt hCC), whereas the proline mutant eluted from the column as two peaks, a major one with R_T = 12.916 min and a minor one with R_T = 15.275 min. The second peak most probably represents the monomeric form of the mutant. The retention time of the first peak is shorter than expected for the dimer of hCC (R_T ≈ 13.3 min calculated based on the calibration of the column) and corresponds to a (globular) protein with a molecular mass of around 32 kDa instead of the expected 26.6 kDa. This discrepancy can be caused by a non-spherical shape of the hCC dimer (which is rather ellipsoidal based on the crystal structure) or an increase of the Stokes radius of this particular mutant due to higher content of the molten globule conformation and overall loosening of the structure. It is known from the literature that the molten globule state can manifest as an increased Stokes radius observed during gel filtration (Ptitsyn, 1995).

Dimerization propensities of hCC variants

Dimerization of wt hCC and its variants was induced by addition of guanidine hydrochloride to 0.5 mg/ml solutions of the studied protein in PBS. Samples were then incubated at 37°C and the dimerization progress was checked using gel filtration and an agarose gel electrophoresis. Quantitation of the amount of the dimer formed was performed using the Empower software (Waters). The hCC variants showed distinct propensities for dimer formation (Fig. 4). At 0.5 M Gdn·HCl concentration, which was routinely used for the induction of dimerization of wild type hCC and its mutants studied by us so far (Nilsson *et al.*, 2004), variants V57D and V57N stayed mostly monomeric throughout incubation (8.1% dimer formed after 9 days for hCC V57D and 7.7%

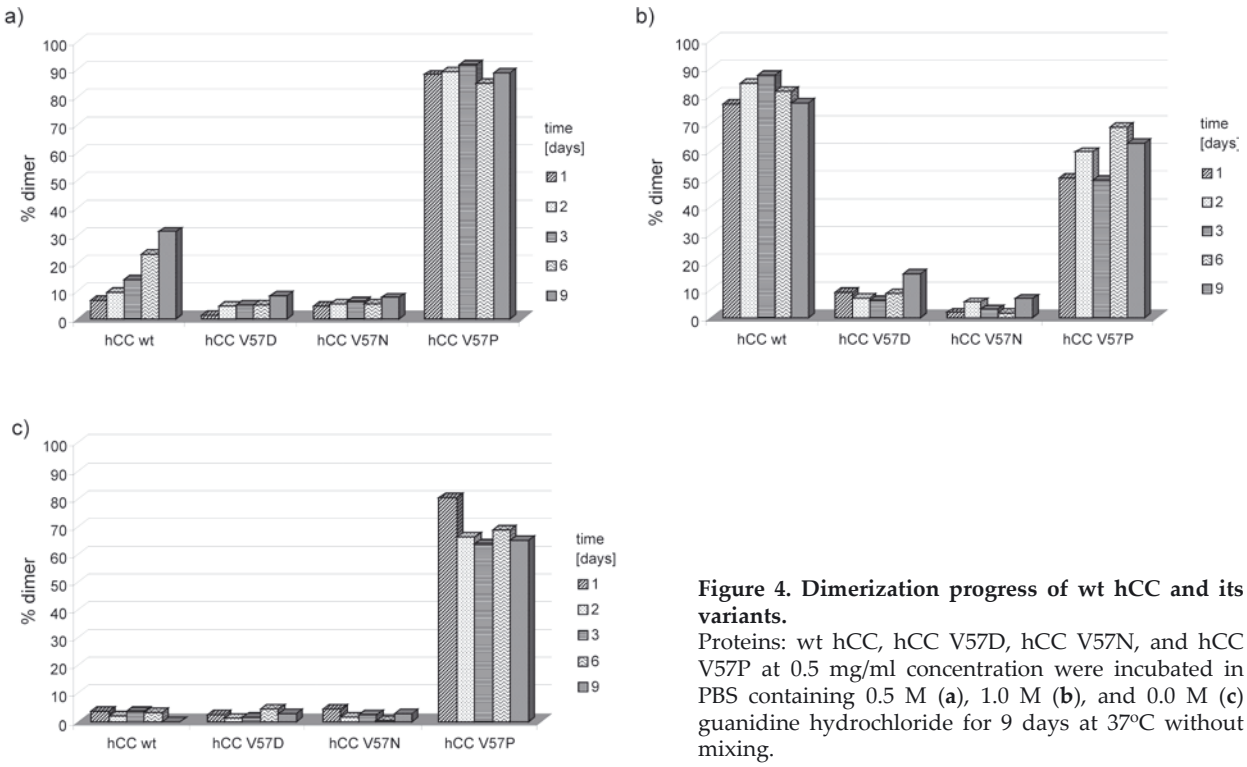

Figure 4. Dimerization progress of wt hCC and its variants.
Proteins: wt hCC, hCC V57D, hCC V57N, and hCC V57P at 0.5 mg/ml concentration were incubated in PBS containing 0.5 M (**a**), 1.0 M (**b**), and 0.0 M (**c**) guanidine hydrochloride for 9 days at 37°C without mixing.

for hCC V57N, Fig. 4a). In contrast, the proline mutant was predominantly dimeric, even when freshly dissolved in the incubation buffer, and the proportion of the dimer only slightly increased with incubation time. This observation is in good agreement with the Bergdoll's suggestion (Bergdoll *et al*., 1996) that proline in the linker region may facilitate the domain swapping, mostly by rigidifying the hinge region and keeping it in an extended form. Widening of the turn can also lead to the weakening of the hydrogen bond network, which may additionally account for the observed tendency of the proline mutant for opening and existing in the swapped form. Wild type cystatin C at the same conditions dimerized in 31%. Increasing of the denaturant concentration to 1.0 M had minor influence on the dimerization propensity of the V57N mutant (Fig. 4b). In the case of V57D the dimer content in the incubation mixture after 9 days doubled comparing to the previous experiment but still remained significantly lower than for the wild type protein and the V57P variant. For the latter protein at 1.0 M Gdn·HCl the equilibration between the monomer and the dimer was observed during the time course of the experiment, which suggests similar stability of both forms of hCC V57P at these conditions. The biggest impact of the denaturant concentration on the oligomeric state of hCC was observed for the wild type protein, which dimerized rapidly after dissolving in the buffer containing 1.0 M Gdn·HCl. The amount of the dimer further increased during the first days of incubation, but in the following days a decrease in the dimer content was observed. Since overall lowering of the protein concentration in the sample was also noted, this suggests precipitation of wt hCC rather than a shift of the equilibrium towards the monomeric form.

The resistance of the V57D and V57N variants to dimerization was also confirmed in an experiment in which the concentration of the proteins was doubled. No change in the dimer content in comparison to the diluted samples was observed for these proteins after 7 days of incubation with 0.5 Gdn·HCl, whereas for wild type and the V57P mutant, which are dimerization-prone or dimeric, an increase in the dimer proportion was noted (not shown). The increased monomeric stability of hCC V57N was recently confirmed by crystallographic studies which showed the presence of two monomeric molecules of this protein in an asymmetric unit (Orlikowska M., personal communication).

Prolonged incubation of all the studied proteins without the addition of any factor causing their destabilization did not change their oligomeric state (Fig. 4c).

DISCUSSION

The results presented in this paper clearly confirm the important role of the L1 loop of hCC in dimerization and, possibly, also in further oligomerization of this protein. The conformational strains, centered on the Val57 residue, can induce

the spring-like behavior of this structural element of the protein, leading to the opening of the molecule and its subsequent association into dimers or higher oligomers. We showed that after the change of the unfavorable valine to a preferred aspartic acid or asparagine residue, the monomeric structure of hCC was preserved even at elevated denaturant concentrations and upon prolonged incubation. On the other hand, introduction of the dimerization-promoting proline residue in position 57 indeed led to the formation of the elevated amounts of the dimer in incubated but also in the untreated hCC V57P variant. Our experimental data are in good agreement with our theoretical calculations (Rodziewicz-Motowidło *et al*., 2009). Changes in the width of the L1 loop and the hydrogen bond network, as well as the influence of a particular mutation on the conformation and dynamics of the loop indeed caused either stabilization of the hCC in the monomeric form (for the asparagine and aspartic acid mutants) or opening of the molecule leading to preferential dimer formation (hCC V57P). A stabilizing role of an Asp residue in the L1 loop was also reported by Staniforth *et al*. (2001). Those authors reported that the Val55 to Asp55 mutation inhibited domain swapping of stefin B. Analogous mutation in the hCC protein also leads to stabilization of the monomeric protein. Our findings for the proline variant of cystatin C also provide further support for the Bergdoll's hypothesis about the role of proline residues in the domain swapping propensity of a particular protein. On the basis of our data and those from the literature, we suggest that the Pro57 residue in the L1 loop of hCC prefers a conformation suitable for dimerization/oligomerization.

The dimerization of hCC takes place at partially denaturating conditions. It was shown before for, e.g., stefin A (Jerala & Žerovnik, 1999) and other cystatins (Ekiel & Abrahamson, 1996; Staniforth *et al*., 2001) that partial unfolding of the protein resulting in the formation of the molten globule state may be the first step in the dimerization process. It was also shown that the dimeric form of cystatins is the stable one whereas the monomer can be treated as a kinetic trap in the folding process (Jerala & Žerovnik, 1999; Rodziewicz-Motowidło *et al*., 2004). In these terms all factors increasing the energy barrier between the monomer and dimer would shift the monomer/dimer ratio toward the former state. This can be accomplished by engineering the "hot spots" in the protein sequence known to influence its stability. In our case we have focused on the L1 loop of cystatin C, proposed to play an important role of a molecular spring facilitating domain swapping. In our studies we increased the protein stability in the monomeric state by individually introducing two mutations that limited the number of possible conformations thus leading to the family of better-defined structures (Rodziewicz-Motowidło *et al*., 2009). In addition, the side-chains of Asp and Asn residues could form hydrogen bonds with other amino acids and by this stabilize the L1 loop in the "closed" form. The hydrogen bonds could not be formed by the Val or Pro side-chains. Therefore, on their way from a partially unfolded to natively folded protein the asparagine and aspartic acid mutants will have to sample a more limited number of conformations leading to the kinetic, monomeric fold.

We also have provided proof for an opposite effect caused by the proline mutation. Introduction of the Pro residue caused lowering of the protein stability, visualized by its lower melting temperature and also by an increased tendency for dimerization. Our theoretical calculations for the β1-L1-β2 fragment of hCC and its mutants showed that the proline mutation gives rise to a much broadened loop L1 and the existence of two conformational families (Rodziewicz-Motowidło *et al*., 2009). The free energy barrier between them is very low (about 2 kcal/mol) which is 10 times less than the value for the *cis-trans* isomerization of the Xaa-Pro peptide bond (Wedemeyer *et al*., 2002). Nevertheless, it cannot be ruled out that in the case of an intrinsically meta-stable protein and in the presence of destabilizing factors (eg., denaturating conditions or elevated temperature) such *cis-trans* isomerization may take place. At this point it is tempting to suggest that the V57P mutant of hCC exists predominantly as a molten globule for which the conversion to the more stable dimeric form is easier than for the other hCC mutants. However, more detailed studies are necessary to verify this hypothesis. Similar consequences of proline introduction, especially, but not only, in the hinge region, on the oligomerization propensity of other cystatins (Jenko Kokalj *et al*., 2007) and other proteins (Bergdoll *et al*., 1997; Rousseau *et al*., 2001; 2003) have been reported.

In conclusion, the results presented in this paper strongly support the hypothesis that the loop L1 plays a role of a molecular spring in the dimerization process of human cystatin C. The conformational strains present in the native protein may not provide sufficient force to cause partial protein unfolding and subsequent formation of a more stable dimer, but together with other destabilizing factors (mutations, environmental factors) they may lead to fast oligomerization and fibrillization, as observed for the L68Q cystatin C mutant. Introduction of a rationally designed mutation in the strained part of a protein has provided valuable proofs that the dimerization and possibly oligomerization process can be controlled to a certain extent by single amino acid substitutions. Rationally designed mutations in the strained part of proteins which exist mainly in

the oligomeric form could have some practical aspects. The specific mutation could allow investigating the biological, biochemical or structural propensities of the monomeric protein. In the case of the studied protein, the V57N mutant, in contrast to the wild type protein, forms in crystallographic experiments monomeric molecule in an asymmetric unit (Orlikowska *et al*., personal communication). Additionally, we are currently studying if the introduced stabilization will provide sufficient force to limit oligomerization of the amyloidogenic mutant of hCC (L68Q).

Acknowledgements

We would like to thank Professor Krzysztof Liberek and his group from the Department of Molecular and Cellular Biology, Inter-Collegiate Faculty of Biotechnology, University of Gdańsk and Medicinal University of Gdańsk, for their great hospitality, all help and providing us with free access to their laboratory equipment. We also thank Dr. Ewa Wieczerzak and Professor Stanisław Ołdziej for stimulating discussion.

This work is supported by the Polish Ministry of Science and Higher Education, grant N204 161 32/4233.

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