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Pressure as a denaturing agent in studies of single-point mutants of an amyloidogenic protein human cystatin C

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ABSTRACT

Recently, we presented a convenient method combining a deuterium-hydrogen exchange and electrospray mass spectrometry for studying high-pressure denaturation of proteins (Stefanowicz et al., Biosci Rep 2009; 30:91–99). Here, we present results of pressure-induced denaturation studies of an amyloidogenic protein—the wild-type human cystatin C (hCC) and its single-point mutants, in which Val57 residue from the hinge region was substituted by Asn, Asp or Pro, respectively. The place of mutation and the substituting residues were chosen mainly on a basis of theoretical calculations. Observation of H/D isotopic exchange proceeding during pressure induced unfolding and subsequent refolding allowed us to detect differences in the proteins stability and folding dynamics. On the basis of the obtained results we can conclude that proline residue at the hinge region makes cystatin C structure more flexible and dynamic, what probably facilitates the dimerization process of this hCC variant. Polar asparagine does not influence stability of hCC conformation significantly, whereas charged aspartic acid in 57 position makes the protein structure slightly more prone to unfolding. Our experiments also point out pressure denaturation as a valuable supplementary method in denaturation studies of mutated proteins.

Proteins 2012; 80:2417–2425.

Key words: unfolding/refolding; hydrogen-deuterium exchange; mass spectrometry; electrospray ionization.

INTRODUCTION

Mechanisms governing pathological aggregation of proteins have not been determined yet. It is generally assumed, however, that in most cases formation of oligomeric assemblies is preceded by partial unfolding of protein molecules, connected with destruction of noncovalent bonds stabilizing their native secondary and tertiary structure. It is then highly desirable to carefully examine an unfolding process of aggregating proteins in order to gain information about factors influencing their stability and structural elements responsible for initiation of unfolding and oligomerization processes.

High pressure is one of perturbants that may influence the equilibrium between folded and unfolded states in protein systems. We have chosen high pressure as a denaturing agent in human cystatin C (hCC) studies for several reasons. First of all, high pressure has already proved to be a suitable tool for tuning and testing protein conformations.[1,2] As a subtle denaturant that does not alter chemical composition of the sample, pressure can induce denatured states in conditions compatible with biological systems.[3] Besides, pressure unfolding usually avoids irreversible aggregation common in heat denaturation, and as a reversible process, allows to follow not only unfolding but also refolding of proteins.[4] In addition, application of specific pressure provides a convenient means to populate and characterize partially folded states, which are thought to have a key role in formation of amyloid fibrils.[5] The mechanism of pressure unfolding is different from a chemical or a heat-induced one. In the contrary to them, it can be described not as exposition of the protein hydrophobic interior to the solvent, but as penetration of water molecules into the hydrophobic core of a protein. According to this mechanism, increasing hydrostatic pressure forces water into the protein cavities,

Grant sponsor: University of Gdansk; Grant number: BW/8000-5-0253-9; Grant sponsor: Polish Ministry of Science and Higher Education; Grant numbers: DS/8440-4-0172-1, 1264/B/H03/2009/37.
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Received 29 December 2011; Revised 16 May 2012; Accepted 28 May 2012
Published online 6 June 2012 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/prot.24126

Figure 1

Crystal structures of (**a**) created through 3D domain swapping dimer of hCC wt, (**b**) hCC Val57Asn mutant, which is the only monomeric structure obtained in crystallization experiments of the studied point-mutants of hCC. The structure illustration was prepared using PyMol [].

leading to swelling of the hydrophobic core, weakening of interactions between nonpolar side chains, and, as a consequence, to unfolding of a protein molecule.[6–8]

hCC is a small (13.4 kDa) natural cysteine proteases' inhibitor, ubiquitous in all body fluids.[9] Its physiological role is vital and is believed to be tightly linked to the control of proteins turnover and defense of tissues against invasions of viruses and parasites.[10,11] Besides its inhibitory function, hCC plays an important role in development of neurodegenerative diseases. Aggregation of the naturally occurring mutated protein—Leu68Gln hCC, evokes autosomal dominant hereditary amyloid angiopathy Icelandic type (HCCAA-I), which results in paralysis, dementia and eventually death of young, in most cases less than 40 years old adults.[12–14] The wild-type hCC is also implicated in a disease connected with pathological aggregation, because it was colocalized with amyloid β in parenchymal and vascular cerebral amyloid deposits of patients with Alzheimer disease.[15]

The three-dimensional structure of the monomeric wild-type hCC has not been determined till now, although it could be predicted from the very recently determined monomeric structures of hCC mutants: Val57Asn (PDB ID: 9NX0[16]) and stab-1 (PDB ID: 3GAX,[17] as well as on the basis of the domain-swapped dimeric structure of the wild-type hCC published few years ago (PDB ID: 1G96[18]) (Fig. 1). Comparison between these structures shows that the dimer reconstructs in duplicate the general fold of the monomeric hCC with the exception of the hinge region, which links the swapping subdomains. This region, encompassing residues 55–59 (Gln-Ile-Val^{57}-Ala-Gly), in the monomeric protein forms a loop called L1, constituting an element of the inhibitory epitope. During the dimerization process the L1 loop undergoes significant structural changes, as a consequence of which it straightens and comes to being a part of the very long β-strand (Tyr42-Thr74). Theoretical calculations have pointed towards the residue at the position 57 as the main source of tension destabilizing the L1 loop in the monomeric hCC molecule.[19] This result encouraged us to design and construct hCC mutants with Val57 residue replaced by aspartic acid (Asp), asparagine (Asn), or proline (Pro).[20] The aim of these mutations was to check if substitution of hydrophobic valine residue by polar asparagine or aspartic acid, preferred in β-turn regions,[21] or proline, recognized as a residue able to destabilize loop structures,[22] would influence the protein stability.

Val57Asn, Val57Asp, and Val57Pro mutants as well as the wild-type hCC were the subject of the presented here studies concerning susceptibility of the proteins to high pressure-induced denaturation. To investigate the pressure impact on unfolding/refolding process of particular hCC variant, we employed hydrogen/deuterium exchange mass spectrometry (HXMS), which is a powerful, sensitive and versatile technique for probing both structure and conformational dynamics of proteins in solution.[23,24] The technique exploits the fact that protons involved in hydrogen bonds or buried in the core of a protein do not exchange readily with deuterons provided by a solvent. Hence, a rate of H/D exchange reflects solvent accessibility: high exchange rates are indicative of a poor structure, whereas decrease in a rate of an exchange suggests a folded structure with restricted access of the solvent to the interior of a protein and involvement of hydrogen atoms in stabilization of secondary structure elements.[25–27] Analysis of pressure dependence of a deuteration level of the wild-type and mutated hCC helped us to confirm significance of Val57 residue located at the apex of the L1 loop for the protein unfolding and probably also oligomerization process. Our work is also a first attempt to study in a systematic way the effects of point mutations on the pressure stability of proteins.

MATERIALS AND METHODS

Reagents

Deuterium oxide (99.9% D), formic acid, acetonitrile and ammonium formate were purchased from Sigma-Aldrich. The wild-type hCC and its mutants were obtained by protein engineering methods.[20]

Deuteration experiment

0.1 mg of the lyophilized powder of each protein was dissolved in 10 m*M* ammonium formate pH 6.9 and incubated for 30 min to allow the proteins to achieve their native conformation. After this time the solutions were 10x diluted by 10 m*M* $HCOONH_4/D_2O$ (final protein concentration: 0.1 mg/mL), what initiated H→D exchange. The obtained diluted samples were immediately applied to MS instrument and for each of them the isotopic exchange was continuously observed for about 60 min.

High-pressure experiment

High-pressure denaturation experiments were carried out according to the protocol established in our previous studies of model proteins.[28] Samples were prepared by dissolving 0.1 mg of the protein in 0.1 mL of 10 m*M* ammonium formate in D_2O (pH = 6.9). The protein solution in a stoppered polypropylene syringe was placed in a custom-made cylinder-piston-type apparatus filled with hexane as a transmission medium. The samples were compressed at room temperature to a given pressure (100–1000 MPa) and incubated under such conditions for 30 min. After decompression 50 μL of each sample was diluted with 450 μL of 10 m*M* ammonium formate in water, what initiated D/H back-exchange under atmospheric pressure. The obtained solution (final protein concentration 0.1 mg/mL) was immediately infused into the ion source of a mass spectrometer, simultaneously with 0.5% formic acid in acetonitrile. Flow rate was 3 μL/min for both solutions.

ESI-MS measurement

All MS analyses were performed on Apex-Qe 7T FTICR spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ion source. Spectra for each sample were collected for at least 90 min. The instrument was operated in the positive ion mode. The capillary potential was set at 4200 V in a spray chamber and 350 V at the capillary exit. Scan range was 300–3000 *m/z*. The instrument was calibrated externally with Tunemix™ mixture (Bruker Daltonics) in a quadratic regression mode.

Calculations of the extent of deuterium incorporation

The number of deuterons in the protein samples was calculated as follows: $D(r) = (M_D - M_0)/a$, where $D(r)$ is the number of deuterons, a is a mass of a deuterium atom expressed in daltons (Da), M_D is the molecular weight of the deuterated protein sample, and M_0 is the molecular weight of the fully protonated protein sample. Values of M_D for each data point were obtained by deconvolution of the recorded spectra using DataAnalysis program provided by Bruker Daltonics.

The correction for the background connected with 10% D_2O presence in the samples used in D→H exchange experiments was also made. The corrected number of incorporated deuterons was calculated by subtracting from each $D(r)$ the number of deuterons present in the sample incubated under ambient pressure in a D_2O-based buffer. The corrected number of deuterons as a function of the applied pressure was presented in Figure 5.

RESULTS AND DISCUSSION

Characteristics of the mutants

The wild-type hCC and its single-point mutants: Val57Asn, Val57Asp, and Val57Pro, were tested in regard to their stability and oligomeric state in solution. Thermal denaturation studies followed by CD measurements showed that hCC wt and the mutants with Asn or Asp residue at the position 57 displayed comparable stability. The melting points for these proteins was similar and equal to 81.0–81.5°C whereas for Val57Pro variant the transition point temperature was lower by about 10°C, clearly indicating lower stability of this mutant (results not shown). Similar differences were observed in the mass spectrometry analysis of the proteins' deuteration processes. After about 60 min of incubation in a deuterated buffer Val57Asn, Val57Asp, and the wild-type cystatin C incorporated 180–181 deuterons, while Val57Pro hCC–189 deuterons (Fig. 2). The difference in the proteins behavior was evident from the very beginning of the deuteration process: after 5 min of incubation more stable hCC variants exchanged 170–171 protons into deuterons while Val57Pro mutant – already 177. Comparison of these numbers with the total number of exchangeable protons present in each molecule (see below), diminished by 10% due to 10% content of the protic solvent in the samples, allows to calculate the number of protons constituting the hydrophobic core of each hCC variant. In the case of Val57Pro variant only 12 protons were protected against the isotopic exchange whereas almost 20 protons were buried in the hydrophobic core of Val57Asp, Val57Asn, and wt hCC.

Val57Pro mutant was different from the rest of the studied proteins also in regard to the displayed tendency to dimerization. Although Val57Asn, Val57Asp, and the wild-type hCC were mainly monomeric when analyzed by size-exclusion chromatography (SEC), in the case of the mutant with Pro residue at the critical 57 position

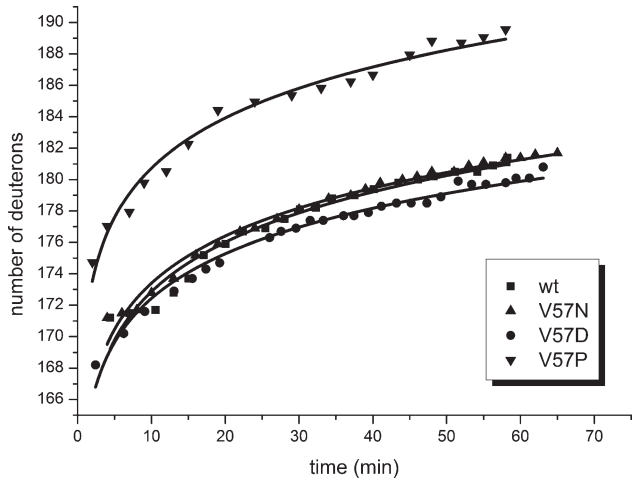

Figure 2

Substitution of protons of hCC wt and its Val57Asn, Val57Asp and Val57Pro mutants by deuterons, under atmospheric pressure, followed by MS analysis. Squares - wt hCC, circles—Val57Asp, triangles—Val57Asn, inverted triangles - Val57Pro.

substantial amount of the dimer was evidenced on SEC chromatogram.[20] By applying special conditions during MS analysis we were also able to detect Val57Pro dimer, however only during the experiments, in which no acidified organic solvent was administered simultaneously with the protein sample.

Experiment design

In our experiments we have used HXMS technique to identify structural perturbations connected with high pressure treatment of an amyloidogenic protein—hCC and its three single-point mutants: Val57Asn, Val57Asp, and Val57Pro. Because ESI-MS analysis can be performed only under atmospheric pressure we employed a special procedure to study influence of elevated pressure on the proteins conformation.[28] In brief, the proteins were dissolved in a deuterated buffer and incubated for 30 min under elevated pressure, what induced a various degree of unfolding and enabled incorporation of a different number of deuterons into the protein molecules. After decompression the proteins started to refold trapping incorporated deuterons in their interior. Dilution of the samples in an aqueous buffer initiated the back-exchange of deuterium atoms located on the protein surface into protons, whereas the deuterons captured inside the refolded protein were protected against D/H exchange process. By determination of the molecular mass of the protein sample by means of mass spectrometry we were able to establish the number of unexchanged deuterons. As the controls served the protein solutions either in a nondeuterated or deuterated aqueous buffer, incubated under atmospheric pressure.

In each experiment we needed 2 min to complete decompression of the deuterated sample, transfer it to an eppendorf tube and dilute in a nondeuterated buffer. From that point, one additional minute was required to apply the sample to MS apparatus and acquire a spectrum of satisfying intensity of the recorded peaks. Because of these technical demands a 3-min time interval separated in all cases the start point of decompression from the first data report taken under consideration. For 1/3 of this time, the samples were already exposed to a nondeuterated buffer, which provided protons to substitute the incorporated deuterons. We were thus not able to determine the exact number of deuterons incorporated to the protein chain as a result of specific pressure application, however, in all cases we started to record spectra at the same time point and could estimate and compare the degree of the isotopic exchange in each sample directly after the 'void' time. Besides, although we followed the kinetics of deuterium-hydrogen back-exchange from the earliest technically possible moment, our conclusions about the pressure impact on stability of different hCC variants were based on the data collected after 60 min. of the sample incubation in a nondeuterated buffer, when the equilibrium of the back-exchange process in most cases seemed to be established.

During application to the mass spectrometer, the protein solution was mixed with 0.5% formic acid in acetonitrile, using T-splitter installed directly before the ESI source. Addition of an organic solvent and acidification of the solution improved an ionization of the protein, significantly enhancing sensitivity of MS analyses. Moreover, decreasing of pH was also supposed to suppress the isotopic exchange,[23,29] which may occur between the T-splitter and ESI-needle.

Exchangeable protons in hCC molecule

hCC as a protein containing 120 amino acids among which eight are Pro residues, possesses 111 amide protons potentially available for hydrogen-deuterium exchange (Val57Pro mutant – 110). Besides the backbone protons there are also labile protons from the amino acid side chains, overall 107 atoms (in Val57Asn and Val57Asp – 109 and 108, respectively), and the protons from the terminal amine and carboxylic groups, which can also be substituted by deuterons. In summary, the total number of the exchangeable protons in the proteins in their native state amounts to 221 in the wild-type cystatin C, 220 in Val57Pro mutant, and 223 and 222 in Val57Asn and Val57Asp variants, respectively.

All hCC variants, incubated in a deuterated buffer at ambient pressure (denoted as 0 MPa) and subsequently equilibrated for 60 min in a solution diluted to contain only 10% of D_2O, exhibited in their molecules a certain amount of the preserved deuterons. Val57Asp and Val57Asn mutants occurred to possess 23 deuterons, Val57Pro – 24, and the wild-type cystatin C – 26 deuterons [Figs. 3 and 4(a)]. When we display these numbers as a ratio between the

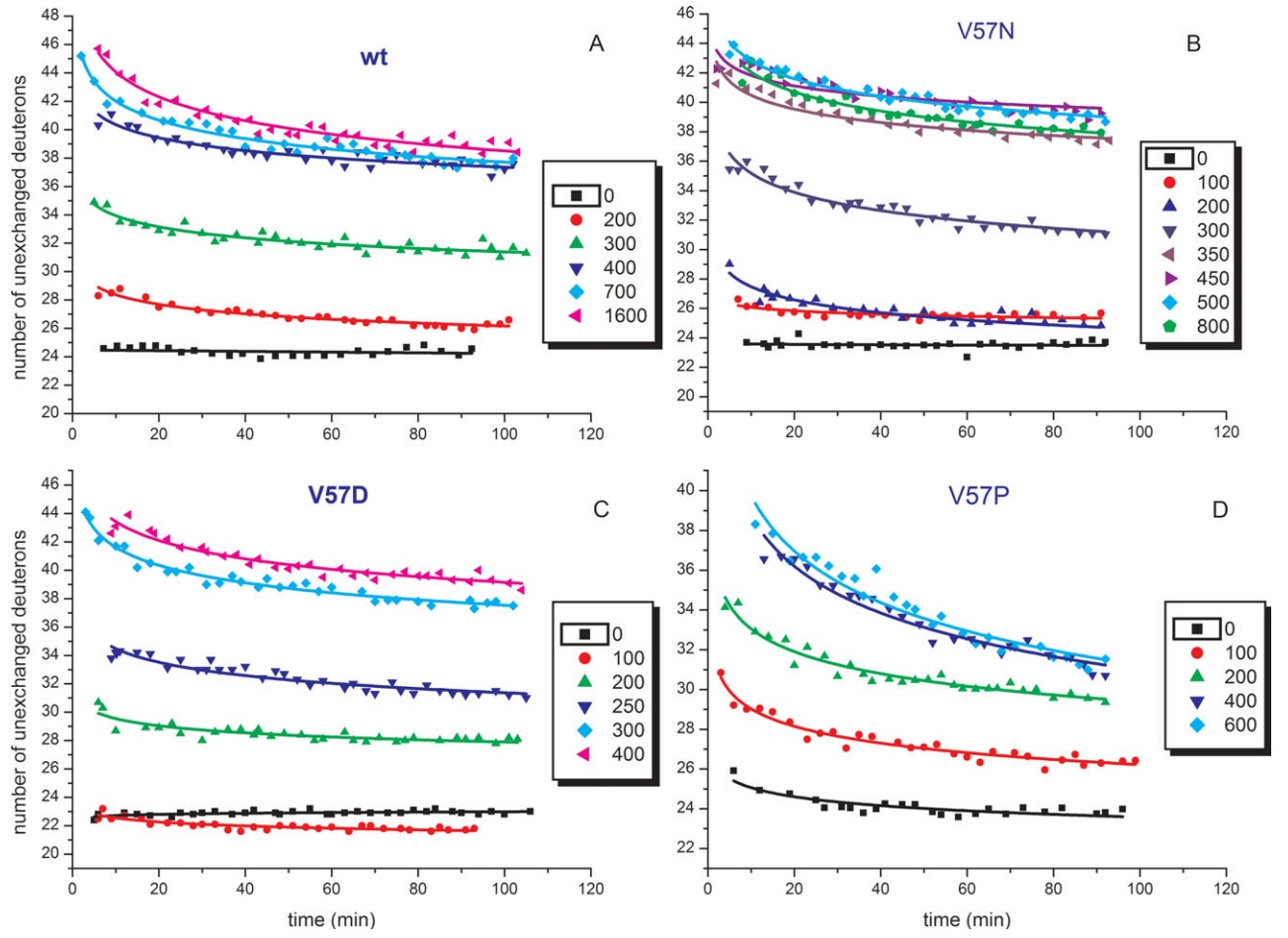

Figure 3

Kinetics of D/H back-exchange in the wild-type human cystatin C (**a**), Val57Asn (**b**), Val57Asp (**c**), and Val57Pro (**d**) mutants, treated with different pressure. Numbers in each plot legend correspond to pressure expressed in MPa. '0' means 'no additional pressure applied', that is incubation of the samples under atmospheric pressure (0.1 MPa).

number of the preserved deuterons (D(r)) and the number of all potentially exchangeable hydrogen atoms, the calculations in the case of each protein yield the similar result of about 10%. This number of deuterons constitutes the background connected with unavoidable, due to the experiment procedure, presence of D_2O in all samples.

Analyzing the obtained results we discriminated between the total number of deuterons detected in the proteins' structure and the number of deuterons, which had been captured in the interior of the refolded molecules. The time-dependent charts (Figs. 3 and 4) in each case present the total number of deuterons, whereas the data in Fig. 5 are corrected for the background connected with 10% D_2O content.

Stability of hCC variants in comparison to lysozyme and ubiquitin

The incipient changes of the protein structure occurred at different pressure values for each of the studied hCC variants [Fig. 3(a–d)]. The number of deuterons as a function of the refolding time was charted for the selected pressure values (the first ones for which more significant change was detected: 300 MPa for hCC wt and V57N, 200 MPa for V57D and 100 MPa for V57P), related to the intermediate, partially unfolded state of each hCC analog [Fig. 4(b)]. The wild-type cystatin C and Val57Asn, one of the two mutants with the substitution supposed to stabilize the L1 loop structure, at the spectra recorded after 60 min of the back-exchange process gave m/z peaks corresponding to incorporation of 31–32 deuterons. At the same time, 28 deuterons were detected as being incorporated to the intermediate conformation of Val57Asp, and only 26 deuterons in Val57-Pro mutant. The outcome of the thermal denaturation studies and the deuteration experiments do not allow to believe that lower number of unexchanged deuterons in the case of Val57Pro mutant results from its greater stability and lower tendency to unfolding. Just the opposite—results of these studies strongly suggest less stable

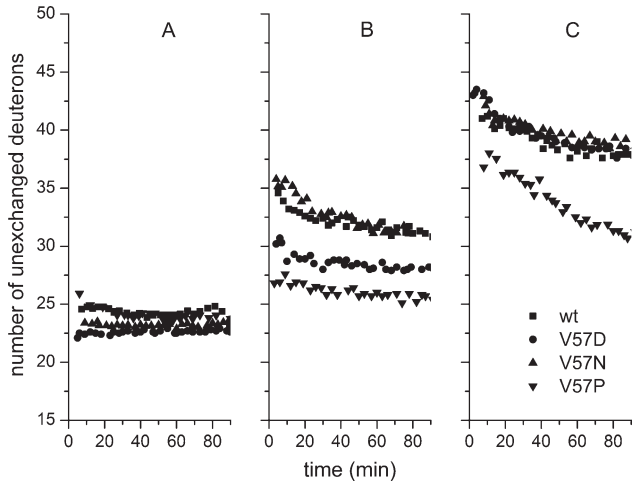

Figure 4

Time-dependent alterations in the number of unexchangeable deuterons in the wild-type cystatin C and its Val57Asn, Val57Asp and Val57Pro mutants. Showed are curves corresponding to (**a**) the beginning (atmospheric pressure, denoted as 0 MPa), (**b**) the intermediate state, (for the wild-type protein and Val57Asn mutant corresponding to 300 MPa pressure, for Val57Asp to 200 MPa and for Val57Pro to100 MPa), and (**c**) the final step of the denaturation process (600 MPa for all proteins). Squares, wt hCC; circles, Val57Asp; triangles, Val57Asn; inverted triangles, Val57Pro.

structure of Val57Pro in comparison to the rest of the tested hCC variants. Detected in the HXMS experiment greater susceptibility of Val57Pro variant to the applied pressure [partial unfolding already at 100 MPa; Fig. 3(d)] also indicates that this mutant is the least stable among the studied proteins. More relaxed structure of Val57Pro would facilitate D→H back-substitution and is probably a reason of smaller number of deuterons which remain unexchanged at the equilibrium state.

At 600 MPa all the studied proteins have already adopted a conformation that could not be further perturbed by the pressure increase [Fig. 3(a–d)]. The final unfolded state does not necessarily mean completely unfolded structure. Pressure denaturation is known as a reversible process allowing proteins to retain some amount of their secondary structure to the very end of the process.[7] However, it is rather not the case for hCC variants since when we examined the protein sample subjected to pressure 600 MPa and then left for 24 h in the deuterated solution, we observed complete deuteration of the protein (results not shown). Besides, resistance of the proteins to unfold further upon application of higher pressure (up to 1600 MPa) also allows to reckon the conformation achieved at 600 MPa to be the final unfolded state.

The wild-type hCC and its Val57Asn and Val57Asp variants, subjected to the pressure 600 MPa at the equilibrium state of the back-exchange process preserved in their structure almost 40 deuterons. Val57Pro mutant after 60 min of D/H back-exchange was able to keep no more than 32 deuterons. (the numbers in this section are not-background-corrected for 10% D_2O presence in the samples). Expressing these numbers as a percentage of all exchangeable hydrogen atoms present in the molecules of the studied hCC analogs, we obtained almost 12% of deuterons protected in the intermediate and about 14% in the final unfolded conformation of Val57Pro mutant. The relevant calculations for the more stable hCC variants, yielded, respectively, 14–15% for the intermediate and 18% for the unfolded conformation.

For the unfolded state of the model proteins, lysozyme and ubiquitin (pressure denaturation studies of which we had previously performed[28]), we had obtained, respectively, 23% of deuterons protected against the back-exchange in the case of containing 76 amino acids (144 labile protons) ubiquitin, and more than 29% for lysozyme consisting of 129 amino acid residues (255 labile protons). For hCC, with its 120 amino acids and 220–223 labile protons, one should expect between 23 and 29% of deuterons preserved in the refolded structure. 18% of deuterium atoms, present in the refolded structure of hCC wt and its Val57Asn and Val57Asp variants, was noticeably less than expected. Analysis of MS spectra of hCC samples unfolded by elevated pressure of 600 MPa, and subsequently diluted not in a protonated but in a deuterated buffer, revealed that in such conditions cystatin C molecules were able to incorporate about 220 deuterons, what means that all exchangeable protons underwent substitution by deuterons. Therefore, the diminished number of deuterons present in the hydro-

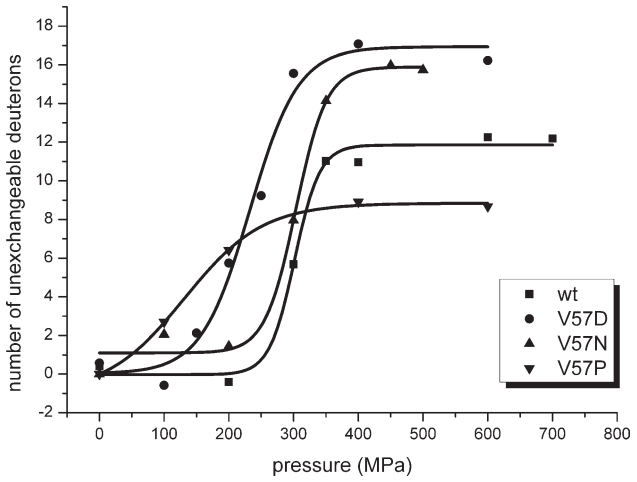

Figure 5

Number of deuterons trapped in the hydrophobic core of the studied proteins, depicted as a function of the applied pressure. Squares, wt hCC; circles, Val57Asp; triangles, Val57Asn; inverted triangles, Val57Pro. The results were background-corrected by subtracting the number of deuterons present in the samples incubated under atmospheric pressure, due to 10% content of deuterium oxide in each of them.

phobic core of the refolded hCC molecules cannot be explained as caused by the protein limited unfolding.

Another possible explanation of the observed discrepancy between the obtained and expected number of the protected deuterons, is an assumption that hCC molecule lost more deuterons on behalf of protons during the refolding process. This interpretation would suggest that cystatin C refolds to its native-like state more slowly than ubiquitin or lysozyme, or that hCC conformation is very labile. Analysis of the samples, which after exposition to the elevated pressure were left in a deuterated buffer overnight to refold, rebuts the first hypothesis since the number of deuterons incorporated to the protein molecule after the prolonged refolding time did not differ from the one obtained in the classically designed experiment.

The answer for the question about smaller than expected number of deuterons captured in hCC molecules, the most likely lies in the protein inherent flexibility. Revealed in deuteration experiments the small number of protons constituting the hydrophobic core of hCC variants, confirms this hypothesis. Propensity of hCC to undergo domain swapping dimerization, which requires existence of the protein in the partially unfolded 'open' state, encourages to believe that hCC may quite easily adopt transiently unfolded conformations. The refolded after pressure relief cystatin C molecule, due to these fluctuating movements, might be able to exchange deuterons previously trapped in the protein interior. In the light of still not fully elucidated mechanism of hCC domain swapping dimerization it would be highly desirable to find out exactly where these deuterons are located. Suitable experiments with the usage of Electron Capture Dissociation (ECD) MS are under the way in our lab.

Differences in pressure impact on hCC variants

Application of elevated pressure to proteins which differ in their conformational stability, should result in a various degree of unfolding and, as a consequence, in a various number of protons available for substitution by deuterons. Plots in Figure 5 present dependence of the number of deuterons trapped by different hCC variants on the applied pressure. The curves are background corrected for 10% D_2O presence in the samples and because of that take into account only the deuterons captured in the interior of the refolded proteins.

In case of Val57Pro lack of any plateau in the first part of its denaturation curve clearly indicates that unfolding of this protein starts at the very beginning of the pressure experiment, confirming that it is the least pressure-stable hCC variant. This mutant is also the most dynamic structure, what is demonstrated by the steeper slope of the curve presented in Figure 3(c). Resulted from the dynamic structure repeating transient expositions of deuterons to the solvent would enable their prolonged reexchange into protons, diminishing the total number of the trapped deuterium atoms, what explains the experimental data. Greater dynamics of Val57Pro conformation, which may be connected with the L1 loop broadening, may also be an explanation why proline mutant dimerizes the most eagerly among all the studied hCC variants. Structure flexibility should facilitate unfolding of the protein molecules and allow to create the 'open monomer' form. Quite easily achievable and fairly stable 'open monomer' may be expected to exist in solution for time long enough to meet another 'open' molecule and swap domains with it, giving rise to the dimeric structure.

The midpoint of the unfolding process, calculated on the basis of the denaturation curve, for Val57Pro is equal to only 150 MPa. The midpoints determined for the wild-type hCC and its Val57Asn mutant are almost identical and reach a value of about 300 MPa, while for Val57Asp variant this value only slightly surpasses 200 MPa. Val57Asp mutant, although less resistant to the growing pressure than the wild-type cystatin C, surprisingly was able to preserve in its interior a higher number of deuterons (Fig. 5). It seems that in spite of its slightly greater susceptibility to unfolding, refolded Val57Asp has less flexible conformation and protects more deuterons against the isotopic exchange. The other mutant with a substitution stabilizing the L1 loop—Val57Asn, also protected more deuterons than the wild type hCC.

Pressure as a denaturing agent

Aggregation of proteins is usually a slow process which, to be studied *in vitro*, needs to be accelerated by application of denaturing factors such as high or low temperature, specific chemicals, or elevated pressure. Various denaturing agents can differently influence the protein aggregation, and fibrils obtained in various conditions are often not identical.[30] It is not obvious then which method is the best to mimic unfolding and aggregation in biological systems, so the most reasonable approach is to test different procedures and compare them.

In denaturation studies of an amyloidogenic protein hCC we decided to check high pressure suitability for detection of differences between the unfolding/refolding processes of hCC single-point mutants. These mutants were supposed to introduce structural modifications in the hinge loop, crucial for the dimerization and probably also higher oligomerization of hCC.[19] Treatment of the studied hCC variants with guanidine hydrochloride, a chemical denaturing agent, increased dimerization tendency in the case of the wild type protein and its Val57Pro mutant, whereas Val57Asn and Val57Asp occurred to remain mostly monomeric.[20] Greater stability of Val57Asn and Val57Asp mutants was expected by us on the basis of the results of theoretical studies[19] as well as

reported resistance of chicken cystatin (cC) bearing mutation Val55Asp (equivalent to Val57Asp in the human counterpart) to the conditions causing denaturation of the wild-type cC.[31] The pressure-induced denaturation experiments of hCC analogs revealed, however, some dissimilarity of Val57Asn and Val57Asp mutants in regard to their conformational stability (Fig. 5). That this difference, although small, is not an artifact convince results of our latest crystallographic studies. Although Val57Asn crystallized as a monomer,[16] Val57Asp repeatedly appeared in crystals as a dimer (PDB: 3SVA; Orlikowska et al., submitted). The mutations, which were designed as stabilizing the loop L1 and, as such, were both supposed to enable maintaining the monomeric fold of hCC, evidently have different impact on the proteins' stability and caused their different response to the crowding conditions in the crystallization solution. The resolved crystal structures of both proteins showed side chains of polar asparagine and charged aspartic acid protruding from the protein surface and proved that they neither creating any special stabilizing bonds with the protein residues nor disturbing any of such contacts. Therefore we believe that a source of the observed diversity in the mutants' behavior is different pattern of hydrogen interactions with the solvent molecules in the case of each protein.

Any distinct differences between Val57Asn and Val57Asp hCC variants were not detected in the proteins chemical denaturation studies nor disclosed themselves in the heat-induced denaturation. It seems therefore that pressure may be a valuable supplement in the unfolding studies of mutated proteins, especially the ones, in which mutation is located at the protein surface and may disturb interactions of the protein with the solvent molecules.

CONCLUSIONS

Results obtained in the pressure-induced denaturation studies of the wild-type and mutated hCC, allow to conclude:

- Single-point mutation in the L1 region can alter compactness of cystatin C molecule and stability of its structure, therefore stabilization of this hinge region may be beneficial in preventing the dimerization and oligomerization of amyloidogenic hCC.
- Stability of Val57Asn mutant is similar to the wild-type hCC, whereas Val57Asp is slightly more prone to unfolding induced by pressure application. The residue with ionic side chain is not able to stabilize the L1 region and the protein monomeric structure as efficiently as polar but not charged Asn residue
- refolded after pressure relief conformation of Val57Asp and Val57Asn mutants is slightly less flexible than conformation of the wild-type hCC
- Val57Pro mutant is quite unstable and its unfolding starts earlier than unfolding of any other studied hCC variant. The smaller number of the unexchangeable deuterons trapped in this protein interior strongly suggests 'open' conformation what is a confirmation of the postulated hCC dimerization mechanism.
- The direct H/D exchange experiments revealed that the wild-type cystatin C and its mutants present dynamic and unstable structures in which not more than 20 amide protons (from the total number 110–111) is protected from the isotopic exchange.
- Our paper is the first example of systematic studies of point mutations influence on the process of the pressure-induced protein denaturation

ACKNOWLEDGMENTS

The authors thank prof. Krzysztof Liberek from the Department of Molecular and Cellular Biology, Inter-Collegiate Faculty of Biotechnology, University of Gdansk, for providing us with the possibility of free access to his laboratory equipment.

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