Folding-related Dimerization of Human Cystatin C*

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With the aim to improve our understanding of the structural basis for protein self-association and aggregation, in particular in relationship to protein refolding and amyloid formation, folding-related processes for human cystatin C have been studied. Using NMR spectroscopy together with chromatographic and electrophoretic methods, a self-association process resulting in dimer formation for protein samples treated with denaturing agents as well as for samples subjected to low pH or high temperature conditions could be studied with amino acid resolution. In all three cases, the dimerization involves properly folded molecules and proceeds via the reactive site of the inhibitor, which leads to complete loss of its biological activity. This dimerization process has potential relevance for amyloid formation by the brain hemorrhage-causing Leu68→Gln variant of cystatin C. The results also indicate that cystatin C dimerization and inactivation may occur in acidified compartments in vivo, which could be relevant for the physiological regulation of cysteine proteinase activity.

The self-association and aggregation of proteins constitutes one of the least understood problems in protein chemistry. Although some insight has been gained in the kinetic aspects of the aggregation process and in the field of its general theory (Glatz, 1992; Honig and Yang, 1995; De Young et al., 1993), very little is known about its structural aspects and about the precise nature of the interactions involved. It is generally assumed that aggregation proceeds via a partially exposed hydrophobic core in a molten globule, but the very general character of the molten globule concept seriously limits any practical consequences. For controlled refolding of recombinant proteins and for stabilization of pharmaceutical formulations, as well as for the development of new therapies for aggregation-related disease states including amyloidosis, more development in this research area clearly is needed.

To add to our understanding of the molecular aspects of amyloid formation, in particular of the specific intermolecular interactions leading to fibril formation, we have started systematic studies of the properties of amyloidogenic proteins. Human cystatin C provides a good starting point for such studies. This small size inhibitor of cysteine proteinases is present in all human body fluids at physiologically relevant concentrations, being most abundant in the cerebrospinal fluid and in seminal plasma (Abrahamson et al., 1986). Although cystatin C in its wild-type form has not been reported to form amyloid in vivo, its Leu→Gln mutated variant (L68Q-cystatin C) is responsible for the dominantly inherited disorder called hereditary cystatin C amyloid angiopathy (Ghiso et al., 1986; Jenasson et al., 1987; Palsdottir et al., 1988). Expression systems for both wild-type and L68Q-cystatin C have been developed (Dalbøge et al., 1989; Abrahamson and Grubb, 1994), and the three-dimensional structure of the human cystatin C-like type II cystatin, chicken cystatin, is well characterized by both x-ray crystallography and NMR spectroscopy (Bode et al., 1988; Engh et al., 1993). The conformation of the 120-residue polypeptide chain of cystatin C is very similar to that of chicken cystatin, with a proteinase-interacting wedge-shaped side involving residues Arg106-Gly111 of the N-terminal segment and two loop-forming segments constituting polyepitope turns in the main, β-pleated sheet structure of the molecule (segments Gln55-Gly59 and around the single tryptophan residue, Trp106). An additional advantage for the study of cystatin C in the aspect of protein aggregation and amyloid formation is the fact that it normally exists in a monomeric form, in sharp contrast to many other amyloid-forming proteins which are found in multimeric, often heterogeneous, and condition-dependent forms (Brange et al., 1987; Brader and Dunn, 1991; Colon and Kelly, 1992).

Only about 15 of the human body fluid proteins are proven to be amyloidogenic (Stone, 1990; Sipe, 1992). The formation of amyloid is often either directly related to specific point mutations or is enhanced significantly by point mutations (Francione, 1991; Glenner et al., 1991; Sipe, 1992). As is particularly clear in the case of transthyretin, many different point mutations which do not form a clear pattern can lead to amyloid formation. Therefore, there is a tendency to explain amyloidogenicity as being caused by reduced stability of the proteins (Hurlie et al., 1994; McCutchen et al., 1993). Earlier results (Abrahamson and Grubb, 1994) suggest that cystatin C and its L68Q variant can follow a similar pattern. In the present investigation, we have undertaken a detailed study of wild-type cystatin C under conditions leading to its folding-related self-association, with the aim to define the molecular background to the events leading to amyloid formation and physiological inactivation of L68Q-cystatin C.

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¶ Throughout this paper, definitions of "self-association" as a reversible process involving the interactions of two or more native protein molecules (with reversible precipitation of the protein as a possible consequence) and "aggregation" as the interaction of two or more denatured protein molecules (which often leads to practically irreversible precipitation) are used, as suggested by Cleland et al. (1993).

1 I. Ekiel, unpublished NMR observations.
Experimental Procedures

Materials—Recombinant cystatin C, with structure and functional properties identical with those of the native inhibitor isolated from human urine, was produced in E. coli and purified using previously described methods (Abrahamson et al., 1988; Dalbge et al., 1989). Superdex™ 75 (preparative grade) resin was obtained from Pharmacia LKB, Uppsaia, Sweden. Guanidine hydrochloride (GdnHCl) was purchased from BDH Chemicals Ltd (Aristar quality), and Z-Gly-p-nitrophosphoramidase from Armand-Frappier, Laval, Quebec, Canada. All other reagents were purchased from Sigma and used without further purifications. Affinity-purified papain was obtained as a gift from Dr. A. C. Storer and R. Dupras.

Preparation of Self-associated Forms of Cystatin C—Self-associated forms of cystatin C were incubated by incubation of 1 mg/mL (75 μM) solution with 50 mM sodium phosphate buffer, pH 6.7, containing 0.1 M NaCl, for temperature-dependent experiments. Experiments at low pH were performed in 0.1 M sodium phosphate buffer or acetic acid solutions, which were prepared in D2O. Samples were incubated for 30 min in temperature experiments and 11–17 h for the pH and GdnHCl experiments. Such treated samples were used directly for NMR experiments as well as for agarose gel electrophoresis. For comparisons of self-associated forms formed after different treatments, GdnHCl solutions or low pH solutions were diluted quickly into a standard phosphate buffer (50 mM phosphate, pH 6.7, containing 0.1 M NaCl), in which no cystatin C self-association was detected even after 3 months of incubation. No conversion of dimers to monomers was observed during storage of diluted samples. Samples incubated in GdnHCl were diluted to a final GdnHCl concentration not higher than 0.1 M and concentrated back to their original volumes using N2 pressure filtration in stirred cells equipped with YM3 or YM10 membranes (Amicon) or in centrifugal microcentrifuges (Centricon 3 or 10; Amicon). The buffer was then further exchanged at least 50-fold. NMR Spectroscopy—Nuclear Magnetic Resonance (NMR) spectra were collected at 500 MHz using Bruker AMX500 and AM500 spectrometers; 10% (v/v) D2O was added for locking purposes to H2O samples. For samples in heavy water, pH values are given as pH-meter readings at 23°C without any further corrections. Chemical shift values are given in parts per million relative to the internal 2,2-dimethyl-2-silapentane-5-sulfonic acid. NMR signals were identified using the assignment performed for the isotope-labeled (15C and 13N) cystatin C under optimal conditions (1–2 ms solutions at pH 6.0, at 30°C). These assignments were extended to the conditions needed in this work by titration-type experiments between the standard conditions and those used in a particular experiment. For most informative high temperature NMR experiments, to verify the assignments, two-dimensional TOCSY experiments with WALTZ-17 or MLEV mixing sequence (Braunschweiler and Ernst, 1983; Bax and Davis, 1985) were performed at 4°C, the highest temperature at which two-dimensional experiments could be performed at 1.5 ms concentrations without samples undergoing dimerization. The mixing time ranged from 40 to 114 ms and the spin decays of 1024 complex points each (120 scans per free induction decay) were acquired. Solvent suppression was accomplished using presaturation. Aromatic ring signals were assigned using NOESY spectra as described elsewhere. In this way, signal assignments in one-dimensional spectra were done for representative amino acids from all parts of the molecule. Most importantly, a good set of assigned signals was obtained for amino acids in the vicinity of the active site (Trp106, Val104, Tyr102, and Ala194).

NMR experiments were run in both regular and heavy water. From the NH signals observed in water, NH of the Trp106 residue is particularly important (see below). In heavy water, as NH signals are exchanged to ND, many aromatic signals can be interpreted clearly. To give an overview of advantages given by both methods and to avoid repetition, results for the GdnHCl experiments will be presented only for the H2O solutions, and results for temperature and pH experiments only for the D2O solutions.

Other Methods—After long incubations at room temperature, cystatin C samples were checked for potential degradation using mass spectrometry (API III MS/MS System, SCIEX, Thornhill, Ontario, Canada). Agarose gel electrophoresis was performed according to Eijpsoon et al.

RESULTS

Cystatin C Self-association under Mildly Denaturing Conditions—From the results of preliminary SEC experiments to find conditions leading to self-association or aggregation of wild-type cystatin C, a self-association or aggregation process was apparent for protein samples treated with denaturing agents, as well as for samples subjected to low pH or high temperature conditions. Moreover, it was noted that the process observed was very slow. It was, therefore, possible to follow it as a function of time using NMR spectroscopy and to trap associated forms by a quick transfer to standard conditions. It was, furthermore, demonstrated by both SEC and NMR spectroscopy that stationary and trapped cystatin C associated forms were very similar, if not identical (data not shown).

Under close to unfolding conditions, the cystatin C associates observed were in an equilibrium with monomers (possibly unfolded), and trapping led to some back conversion to monomers. However, this conversion was sufficiently slow to allow samples under partially denaturing conditions to be analyzed by agarose gel electrophoresis in nondenaturing buffer.

Screening of Conditions Leading to Cystatin C Self-association—Agarose gel electrophoresis was selected as a method for screening of conditions under which cystatin C self-association occurs, as it has been seen earlier in a study of the Leu68→Gln mutated cystatin C (L68Q-cystatin C) that this method is useful for simultaneously detecting dimer formation (as a distinct band shift) and higher order aggregates leading to precipitation (Abrahamson and Grubb, 1994). In Fig. 1, results are shown demonstrating wild-type cystatin C self-association induced by high temperature (a), by guanidine hydrochloride (b), and by low pH (c). In all three experiments, a region of extensive self-association was observed, with a profile resembling that of the frequently seen aggregation of partially unfolded proteins (Ghelis and Yon, 1982; Mitraki and King, 1989). The main cystatin C self-association product was small, homogeneous, and soluble at 75 μM concentration according to the agarose gels. Extensive precipitation was observed only in the
temperature dependence experiment above 80 °C, where cystatin C unfolds, as was evident from NMR spectra run at high temperatures (data not shown). The small self-associated form seemed to have reduced solubility compared with the monomeric molecule, as further aggregation and precipitation were observed in repeated experiments at cystatin C concentrations above 0.4 mM (not shown).

In the experiments with increasing concentrations of guanidine hydrochloride, self-associated cystatin C started to appear at 0.3–0.4 M GdnHCl, and the amount of trapped self-associated form started decreasing at 1.2 M GdnHCl. Similar results were obtained at low pH values, with maximum amounts of self-associated cystatin C observed in the pH range 3.0 to 4.4. The relative amount of self-associated cystatin decreased rapidly when pH was lowered further (Fig. 1c).

Indications for Formed Cystatin C Self-associated Species Being Dimers by SEC—In parallel to electrophoretic analysis of cystatin C samples under different conditions, SEC experiments were performed on selected samples. As seen from the chromatograms in Fig. 2, samples indicated to contain partially self-associated cystatin C by agarose gel electrophoresis, from either the temperature, pH, or GdnHCl experiments, all gave two peaks in SEC, with retention times of 25.0 min (self-associated form) and 30.7 min (monomeric cystatin). The retention time of the first peak was very similar to that of chymotrypsinogen (M_r 25,000) and, therefore, likely represented a cystatin dimer. No soluble species larger than dimers could be detected. However, some insoluble, precipitated material was produced, especially in samples incubated at high temperatures. In the SEC experiments, it was observed that monomeric cystatin C was more retained than the similarly sized protein, ribonuclease A. This excessive retention of cystatin C is caused by interactions with the matrix, most likely via the inhibitor’s proteinase binding site, as a cystatin C variant with the single amino acid substitution Trp106→Gly (W106G-cystatin C) (Hall et al., 1995) gave a retention time of 28.4 min, i.e. quite different from that for wild-type cystatin C, but similar to that for ribonuclease A (28.7 min). The wild-type cystatin C-matrix interaction clearly changes in the dimeric state, as the retention times for wild-type and W106G-cystatin C dimers were identical. The conclusion that the self-associated species studied were dimers was additionally supported by nondenaturing PAGE (data not shown).

NMR Data for Cystatin C Dimerization Induced by GdnHCl—NMR spectra of cystatin C (75 μM) were run as a function of the GdnHCl concentration (Fig. 3). It is clear from these spectra that the dispersion of signals characteristic for folded proteins is preserved up to 1.0 M GdnHCl. In particular, a group of upfield shifted methyl group signals indicate a retained tertiary structure. Also, three methyl group signals of methionine residues retain the chemical shifts of a properly folded, monomeric protein and collapse only at higher concentrations of between 1 and 2 M GdnHCl. In the NH region, downfield shifted signals, typical for a β-sheet structure, are preserved up to 1 M GdnHCl, but change dramatically between 1 and 2 M GdnHCl, supporting unfolding of the protein in this concentration range of denaturant. The NMR results moreover demonstrate a clear transition region between 0.5 and 1 M GdnHCl, where protein conformation seems to be retained, but specific changes are observed for some amino acid residues, including Trp106 and Val104 (a more specific description of these and other NMR changes in relation to the conformational changes will be presented elsewhere). It is important to note that before the protein starts unfolding, the NH proton of Trp106 shows two discrete signals, shifted by 0.16 ppm, indicating existence of a transition between two states. Comparison with Fig. 1 demonstrates a correlation between the formation of the cystatin C dimers and the presence of the characteristic signal shifts most clearly seen for Val104 and Trp106 (Fig. 3). Dimers as detected by agarose electrophoresis are not present in the denaturant concentration of 2.0 M, for which concentration NMR indicated unfolding of the protein. Additionally, in the NMR spectra, an overall broadening of the signals is evident in the 0.5–1.0 M GdnHCl concentration range, consistent with a self-association process taking place.

NMR Studies of pH-induced Cystatin C Dimerization—NMR
NMR Data for Temperature-induced Cystatin C Dimers—At elevated temperatures, NMR spectra of proteins generally exhibit motion-induced increased resolution. A more detailed analysis of cystatin C spectra at high temperatures was therefore carried out, as additional information about the nature of the self-associated forms could be gained. In order to study the dimerization events, special conditions were chosen to minimize higher aggregation (by using a low protein concentration of 75 μM and only a moderately high temperature of 62 °C). Under these conditions, dimerization was slow (hours), and, as an additional advantage, the important signals of branched amino acid methyl groups are well dispersed. To be able to interpret spectra under these conditions, assignments from low temperature NMR at 30 °C were extrapolated by running temperature dependence of one-dimensional spectra (Fig. 5B) as well as two-dimensional TOCSY (Fig. 5A) and NOESY (not shown) spectra at 40 °C. In the one-dimensional spectra, 13 upfield shifted methyl groups from eight amino acids can be followed, as can methyl groups of the three methionine and some other alanine and threonine residues. All together, signals of 21 amino acids could be assigned easily at high temperature (see Fig. 6 and Table 1). Although some of these signals undergo non-negligible shifts as a function of temperature, most of them were below 0.06 ppm in the aliphatic and even smaller in the aromatic region (spectra not shown). They were directly correlated to temperature changes and did not change with time; therefore, they could be attributed to small conformational changes and/or increasing motion in the molecule. At 62 °C, cystatin C clearly preserves its folded structure. After longer (overnight) incubation, dimerization occurs.

As can be seen in Fig. 6, A and B, NMR spectra of the monomeric and dimeric cystatin C are very similar. In particular, most of the characteristic signals which could be assigned easily and followed at high temperature were essentially not changed during dimerization. That includes amino acids in the β-sheet (Tyr62, Leu68, Leu47, Met110, and Phe99) as well as in the α-helix (Tyr34 and Ala30). Also unchanged are Tyr42 and Met41 in the loop between the α-helix and the β-sheet, Leu91 in the loop area of the β-sheet, as well as all histidines and most of the alanine methyl groups. The overall envelope, especially of the aromatic and α-protons of the β-sheet, also remains the same. That is in contrast to Trp106, Tyr102, and Val104 signals, which undergo relatively large (above 0.2 ppm) changes. These latter changes develop slowly, on the time scale parallel to the formation of dimers (data not shown). Similarly, time-dependent changes were detected for the Ala58 methyl group signal.

Comparison of Dimers Formed under Different Conditions—To verify that the cystatin C dimers formed under different conditions are identical, dimers were produced under optimal conditions for each of the three methods described above, and analyzed after buffer exchange. These dimer preparations gave identically migrating agarose gel electrophoresis bands and displayed identical retention times in SEC experiments (Fig. 2). The most detailed comparison was made using NMR spectroscopy. Fig. 6 (spectra b, c, and d) compare the upfield-shifted methyl group and aromatic regions of the proton NMR spectra for the three dimers. For the best comparison,
high temperature (62 °C) was selected for the optimal resolution and separation of signals in the aliphatic region (see Fig. 5). It was tested in an independent experiment (not shown) that at 62 °C during the time period for NMR data collection (2 h) only about 5% of the monomer was converted to dimer and that spectra of dimers practically do not change with time under these conditions. Fig. 6 shows a very close similarity of the spectra in both the aromatic and methyl group region, therefore supporting that all three differently formed dimers are structurally very similar. NMR spectra of three dimeric samples were also very similar at lower temperatures (between 30 and 60 °C), providing an additional support for the similarities of structures.

Dimer Formation Leads to a Loss of Cystatin C Inhibitory Activity against Cysteine Proteinases—X-ray crystallography results for the avian homologue to human cystatin C, chicken cystatin, as well as for the more distantly related human inhibitor, stefin B (also called cystatin B) in complex with papain (Bode et al., 1988; Stubbs et al., 1990) indicate that cystatins generally interact with cysteine proteinases via a contact area involving a very hydrophobic surface built from two loop-forming segments (comprising amino acids QIVAGVN and YAVPWQGT, at positions 55–61 and 102–109, respectively, for human cystatin C). The NMR results shown above for cystatin C residues in these two segments clearly indicate either some conformational changes in the contact region of the molecule or direct interactions via the proteinase-interacting hydrophobic surface. Therefore, it seemed likely that upon dimerization cystatin C would lose its activity as a cysteine proteinase inhibitor. To examine this possibility, dimers were trapped by buffer exchange and purified using size exclusion chromatography, and their activity was assayed against papain (an enzyme with $K_i$ for the interaction with monomeric cystatin C in the picomolar range). Under conditions resulting in a mixture of monomeric and dimeric cystatin C upon trapping, the papain inhibitory activity of the mixture was proportional to the amount of the monomeric form of cystatin C. The monomeric form purified by SEC from such mixtures was in every aspect indistinguishable from freshly isolated (monomeric) cystatin C (NMR spectra, $R_F$ values at SEC, full activity against papain). Incubation times of the papain-cystatin C dimer mixture were varied for up to 1 h, with no signs of inhibition developing. From this result it was concluded that the cystatin C dimer-
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Table I

| Residue | NH  | Hα  | Hβ  | Others |
|---------|-----|-----|-----|--------|
| Ala30   | 8.49| 3.18| 0.51| Hδ 6.96; Hε 6.77 |
| Tyr34   | 8.70| 4.03| 3.28| 3.08 |
| Ala37   | 7.21| 4.33| 1.35|       |
| Tyr42   | 8.11| 4.80| 2.79| 6.82; 7.24 |
| His43   | 8.79| 4.46| 2.77| 2.59 |
| Ala46   | 8.94| 4.36| 1.21|       |
| Leu47   | 9.40| 4.28| 1.36| 1.21 |
| Ala58   | 8.20| 4.72| 1.53|       |
| Val60   | 8.71| 4.68| 1.67| Hγ 0.76; 0.23 |
| Tyr62   | 9.57| 5.09| 2.74| 2.71 |
| Leu69   | 9.58| 4.92| 1.44| 1.17 |
| Leu80   | 8.08| 4.23| 1.28| 0.87 |
| Phe95   | 8.05| 4.18| 3.19| 2.73 |
| His96a  | 8.10|     |     | Hδ 7.17; Hε 7.35; Hζ 7.02 |
| His96b  | 7.95|     |     | Hδ 7.38; Hε 6.09 |
| Leu91   | 7.06| 4.02| 0.87| 0.19 |
| Ala95   | 8.66| 5.03| 1.30| Hγ 0.42; Hδ 0.60; 0.53 |
| Phe99   | 9.56| 5.53| 3.05| 2.84 |
| Tyr102  | 9.10| 5.56| 3.02| 2.74 |
| Val104  | 7.49| 3.79| 1.68|       |
| Trp106  | 6.95| 4.62| 3.49| 3.38 |

* Tentative assignment.

Data were acquired at 40 °C, chemical shifts were referenced to internal 2,2-dimethyl-2-silapentane-5-sulfonic acid.

The aggregation and precipitation during refolding of proteins produced as inclusion bodies constitutes one of the major obstacles in efficient production of recombinant proteins in occurring. All NMR results clearly indicated that the pretransitional region observed for cystatin C is completely different from the molten globule type of an intermediate state, which is quite common in protein folding paths and is often associated with an increased aggregation (Dolgikh et al., 1981; Kuwajima, 1989; Ptitsyn et al., 1990; Ghe´lis and Yon, 1982). NMR spectra of proteins in a molten globule state show features characteristic of a loss of the tertiary structure, as was well characterized for α-lactoglobulin (Dolgikh et al., 1985; Baum et al., 1989).

Such spectra are more similar to those of the unfolded than to the native protein. On the contrary, NMR spectra of the dimeric cystatin C are very similar to those of the native monomeric form, with the majority of the tertiary structure clearly preserved. Although at the current stage of the spectral analysis there is a possibility of changes in other parts of the molecule, the majority of observed shifts in proton signals clusters around the proteinase binding site. Therefore, one can postulate that in approaching the unfolding point, the structure of the protein becomes more loose, which leads to conformational or hydration changes in the loops forming the proteinase binding site. The most evident differences in one-dimensional NMR spectra between the monomeric and dimeric form of cystatin C are in the second hairpin loop (for residues Tyr102, Val104, and Trp106), which according to computer docking of chicken cystatin with papain (Bode et al., 1988) should be within van der Waals contact with the proteinase. Either conformational changes in the active site directly, or dimer formation via the binding site loops, can explain the observed loss of the activity of cystatin C as an inhibitor of cysteine proteinases upon dimerization. The Kᵢ value for cystatin C interacting with papain is in the picomolar range (Abrahamson et al., 1986), and the equilibrium constant for the self-association of cystatin C is in the micromolar range according to our present results. Still, dimerized cystatin C was unable to inhibit papain even after prolonged incubations, which must be explained by the cystatin being trapped in a dimeric form that is separated by a high energy barrier from the monomeric state (details about the barriers will be presented elsewhere).

The aggregation and precipitation during refolding of proteins produced as inclusion bodies constitutes one of the major obstacles in efficient production of recombinant proteins in unfolding under which conditions unfolding of the protein was occurring. All NMR results clearly indicated that the pretransitional region observed for cystatin C is completely different from the molten globule type of an intermediate state, which is quite common in protein folding paths and is often associated with an increased aggregation (Dolgikh et al., 1981; Kuwajima, 1989; Ptitsyn et al., 1990; Ghe´lis and Yon, 1982).
bacterial systems (Cleland, 1991; Mitraki and King, 1989). These problems were studied extensively (Ghélis and Yon, 1982; Mitraki et al., 1987; Brems, 1988; Lehrman et al., 1991; Cleland and Wang, 1990), with the general conclusions that folding intermediates with a partially exposed hydrophobic core are responsible for the aggregation and precipitation (Wetzel, 1992; Mitraki and King, 1989). The dimerization of cystatin C (leading to precipitation at higher concentrations) resembles other systems by exhibiting a characteristic trough (Ghélis and Yon, 1982) under conditions directly preceding unfolding. However, there is a distinct difference, as both the tertiary and secondary structure of cystatin C are conserved under such conditions. As mentioned above, the unusual large hydrophobic patch on the protein surface shows large changes in the NMR signals and, likely, participates directly in the self-association event.

It is clear from the results of the present study that cystatin C can undergo dimerization under conditions when the protein seemingly has a “normal” conformation, quite far from those leading to unfolding. It is possible that other proteins which self-associate or display problems during refolding may follow this case directly. Potentially pretransition-related changes were reported recently for the reverse transcriptase from HIV (Wright et al., 1994), and, in the past, many other proteins were reported having pretranslational changes (Privalov, 1979). Similar mechanisms of interaction could play a role in folding of multidomain or oligomeric proteins. Further studies are needed to characterize such a way of self-association, and cystatin C seems to be an ideal model case for such work.

A mutated variant of cystatin C, L68Q-cystatin C, has been identified as the cause for the genetic disease that is known as hereditary cystatin C amyloid angiopathy or cerebral hemorrhage with amyloidosis, Icelandic type. The Leu68 → Gin substitution in cystatin C results in massive systemic deposits of the protein, in particular in brain blood vessels, leading to hemorrhages and early death (reviewed by Jenson et al. (1987)). Recently, L68Q-cystatin C has been produced by E. coli expression (Abrahamson and Grubb, 1994), and it was shown that the mutated variant precipitates rapidly at human body temperatures, with transitory formation of dimers. Although more work on binding of the L68Q variant of cystatin C is needed, most likely formation of its dimers is similar to that observed in the pretranslational region for the wild-type protein. However, dimers of L68Q-cystatin C are formed at temperatures nearly 30 degrees lower than needed for the wild-type cystatin (Abrahamson and Grubb, 1994). Therefore, one can postulate that the Leu68 → Gin substitution lowers the transition temperature for the unfolding. That would be expected, as it is common that a substitution of an amino acid in the hydrophobic core of a protein decreases its stability (Pacula and Sauer, 1989; Alber, 1989). Results presented in this work suggest that cystatin C provides another system where decreased stability of a mutant protein correlates with its amyloidogenic nature. Increased propensity for the self-association in the broad range before unfolding may have direct relevance for the formation of the amyloid. The wild-type protein should be quite stable under physiological conditions; however, the L68Q variant should be in the self-association prone, pretransitional region. It remains to be determined if partial unfolding of the protein is necessary for the nucleation step and if dimers are indeed on the path to the fibril formation. Since we have found similarities between the behavior of L68Q and wild-type cystatin C under partially denaturing conditions, the next relevant question to address must be a structural comparison between the dimers and exploration of the role of these dimers as potential intermediates in the amyloid formation.

It also remains to be clarified if dimer formation has any physiological significance for wild-type cystatin C, especially in pathological states. The most likely possibility for a physiological dimerization would be in acidic compartments in cells, involved in the endo- and exocytosis. For example, pH in lysosomes is in the 4.6–5.0 region, which corresponds to the range where cystatin C dimerizes to some extent according to our in vitro results. Even lower pH values, corresponding to those resulting in extensive dimerization and parallel inactivation of cystatin C in vitro have been observed beneath adherent macrophages and osteoclasts (Silver et al., 1988). It is important to keep in mind that our in vitro experiments demonstrate that cystatin C is capable of “remembering its history,” as the dimeric inactive form is very easily trapped for amounts of time practically infinite on the physiological time scale. In this context, it is intriguing that inactive cystatin C dimers quite likely similar to those described in this paper are present in lysates of human neuroendocrine cells in which cystatin C is stored in secretory granules together with neuropeptides. Also, cathepsins B and L have been reported to be responsible for the extracellular process of bone resorption (Delaissé et al., 1984; Kakegawa et al., 1993), even though cystatin C is ubiquitously extracellularly and a potent inhibitor of cathepsin B and L activity (Abrahamson et al., 1986, 1990). This apparent conflict could be explained by a dimerization-caused inactivation of cystatin C in the sealed off and acidified compartment under the bone-degrading osteoclasts.

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