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Hinge-loop mutation can be used to control 3D domain swapping and amyloidogenesis of human cystatin C

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ABSTRACT

Cystatins are natural inhibitors of cysteine proteases, enzymes that are widely distributed in animals, plants, and microorganisms. Human cystatin C (hCC) has been also recognized as an aggregating protein directly involved in the formation of pathological amyloid fibrils, and these amyloidogenic properties greatly increase in a naturally occurring L68Q hCC variant. For a long time only dimeric structure of wild-type hCC has been known. The dimer is created through 3D domain swapping process, in which two parts of the cystatin structure become separated from each other and next exchanged between two molecules. Important role in the domain swapping plays the L1 loop, which connects the exchanging segments and, upon dimerization, transforms from a β-turn into a part of a long β-strand. In the very recently published first monomeric structure of human cystatin C (hCC-stab1), dimerization was abrogated due to clasping of the β-strands from the swapping domains by an engineered disulfide bridge. We have designed and constructed another mutated cystatin C with the smallest possible structural intervention, that is a single-point mutation replacing hydrophobic V57 from the L1 loop by polar asparagine, known as a stabilizer of a β-turn motif. V57N hCC mutant occurred to be stable in its monomeric form and crystallized as a monomer, revealing typical cystatin fold with a five-stranded antiparallel β-sheet wrapped around an α-helix. Here we report a 2.04 Å resolution crystal structure of V57N hCC and discuss the architecture of the protein in comparison to chicken cystatin, hCC-stab1 and dimeric hCC.

1. Introduction

Human cystatin C (hCC) is a basic, low molecular mass protein comprised of 120 amino acid residues (MW 13,343 Da). It belongs to a family of single-chain reversible inhibitors of papain-like (C1 family) and legumain-related (C13 family) cysteine proteases (Turk and Bode, 1991). Human cystatin C can be found in many tissues and body fluids, where it plays its physiological role as a high-affinity inhibitor of cathepsins B, H, K, L and S (Grubb, 2000; Henskens et al., 1996). Three regions in hCC sequence, namely the N-terminal segment (S^1–V^{10}) and two hairpin loops, L1 (Q^{55}IVAG) and L2 (P^{105}W), aligned in a wedge-like fashion, are implicated in interactions with the target enzymes.

Besides its physiological inhibitory function, hCC is also involved in neuropathological disorders connected with amyloid deposition. A naturally occurring single-point mutation in cystatin C gene, changing L68 into Q68, leads to hereditary cystatin C amyloid angiopathy, Icelandic type (HCCAA-I), also known as hereditary cerebral hemorrhage with amyloidosis (Gudmundsson et al., 1972). L68Q hCC variant oligomerizes very easily, even at physiological temperature, forming fibrilar aggregates, which deposit in cerebral and spinal vasculature causing recurrent hemorrhagic strokes leading to serious brain damage and death of patients in their early adult life (Gudmundsson et al., 1972). The wild-type human cystatin C shows higher stability as a monomeric protein, however, it is also involved in pathophysiology of an amyloid disorder, co-precipitating together with amyloid β fibrils in the brain of Alzheimer disease patients (Levy et al., 2001).

The general fold of biologically active, monomeric inhibitors of the cystatin family for a long time has been defined only by the crystal structure of chicken egg white cystatin (Bode et al., 1988). The very recently published structure of hCC-stab1, the mutant with an engineered disulfide bridge connecting residues 47 and 69, revealed for the first time an architecture of the monomeric

Abbreviations: AS, appending structure; HCCAA-I, hereditary cystatin C amyloid angiopathy Icelandic type; hCC, human cystatin C; rmsd, root mean square deviation.

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human cystatin C, and confirmed that the human inhibitor displays known from its chicken counterpart canonical cystatin fold with a long α-helix running across a large five-stranded antiparallel β-sheet (Kolodziejczyk et al., 2010). Formed through a 3D domain swapping dimers of the wild-type human cystatin C (Fig. 1a) re-create in duplicate the structural topology of the monomeric protein. Each monomer-resembling part of the dimeric hCC consists of two segments: β1–α–β2 and β3–β4–β5, contributed by different molecules (Janowski et al., 2001, 2004, 2005). The inhibitory loop L1, connecting the exchanging segments, undergoes significant conformational transition upon dimerization, transforming from a β-turn into a part of an extended β-strand.

Connection between cystatin C domain swapping dimerization and its fibrilization has not been elucidated unambiguously till now, nevertheless there are some experimental data indicating that the domain swapping process could be the basis of the formation of cystatin C oligomers and fibrils. Wahlbom et al. demonstrated that cystatin C formed pre-fibrilar oligomers exchanging domains in a propagated manner (Wahlbom et al., 2007). The same authors showed also that variants of monomeric cystatin C stabilized by engineered disulfide bridges clasping together the swapping domains did not produce oligomers (Nilsson et al., 2004). These results suggest similarity of the mechanisms responsible for creation of dimers and higher oligomers, what allows to expect that precise recognition of the molecular spring of cystatin C instability resulting in its dimerization may help to elaborate the strategy, which makes possible inhibition of hCC oligomerization and fibrillization.

Experimental (Engh et al., 1993; Martin et al., 1995) and theoretical (Dehouck et al., 2003; Rodziewicz-Motowidło et al., 2009) studies revealed that the L1 region of cystatin C is conformationally unstable with the largest component of instability related to the residue V57, located at the apex of the loop. The values of ψ angles for this residue are not optimal (Rodziewicz-Motowidło et al., 2009), what can explain propensity of the protein to undergo domain swapping. It agrees with the literature reports linking presence of flexible hinge loops in a protein structure with its ability to swap domains (Ding et al., 2006). In order to assess an influence of residue 57 on the dimerization of hCC, we obtained mutated cystatin C with hydrophobic valine residue replaced by polar asparagine residue, known to be favored in β-turn regions owing to its ability to stabilize turns (Wilmot and Thornton, 1988). The inhibitory activity of this mutant towards papain was found undistinguishable from the wild-type cystatin C, despite the fact that the modified residue is a part of the inhibitory epitope. Performed experiments confirmed predicted stabilizing effect of V57N mutation. In contrary to the wild type, mutated variant of hCC does not undergo dimerization induced by growing concentration of destabilizing agents, e.g. guanidinium hydrochloride (Szymanska et al., 2009). The substitution made the protein also more resistant to the applied pressure and elevated temperature (Jankowska et al., 2009, in preparation) as well as diminished its propensity for oligomerization (Szymanska et al., 2009).

Crystallization of V57N resulted in the monomeric structure (Fig. 1b). This result is very interesting considering the limited scope of the structural intervention in this mutant. In particular, this is the first case when the single-point mutation stabilizing the L1 loop was sufficient to preserve human cystatin C molecule in its monomeric form. By comparison, the only other known monomeric structure of hCC, that is hCC-stab1 is a double mutant, in which the engineered disulfide bridge was intended to abolish domain swapping dimerization by holding the domains together.

We describe the crystal structure of monomeric hCC V57N and discuss the differences between its fold and the structure of chicken egg white cystatin, hCC-stab1 and dimeric human cystatin C.

2. Material and methods

2.1. Protein expression and purification

hCC V57N was overexpressed and purified to homogeneity using a modified procedure elaborated by Szymanska et al. (2009). Briefly, *Escherichia coli* C41(DE3) strain was used for the periplasmic expression of the target protein from the pHD313 plasmid, constructed by Abrahamson and Grubb (1994). The single-nucleotide mutation corresponding to the V57N substitution was introduced into this construct by means of PCR-based oligonucleotide-directed mutagenesis using the thermostable Pfu DNA polymerase (Fermentas), according to the manufacturer protocol. Protein expression was induced thermally, by quickly rising the culture temperature to 42 °C and then lowering it to 40 °C for a 3-h incubation. The harvested bacteria were re-suspended in a buffer containing 20 mM Tris, pH 7.5, 10 % (v/v) glycerol and flash-frozen. Protein was isolated using repeated freeze/thaw treatment followed by classic cold osmotic shock protocol (Neu and Heppel, 1965). Appropriate fractions were collected and purified by ion-exchange chromatography on S-Sepharose using a linearly increasing salt concentration gradient (0–0.5 M NaCl in 20 mM Tris, 1 mM benzamidinium chloride, pH 7.5) as an eluent. Fractions containing pure hCC V57N were pooled, extensively dialyzed against 10 mM ammonium bicarbonate, pH 8.0, and lyophilized. The lyophilized protein was dissolved in 20 mM ammonium bicarbonate buffer, 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 protein was confirmed using SDS–PAGE electrophoresis, gel filtration, and mass spectrometry.

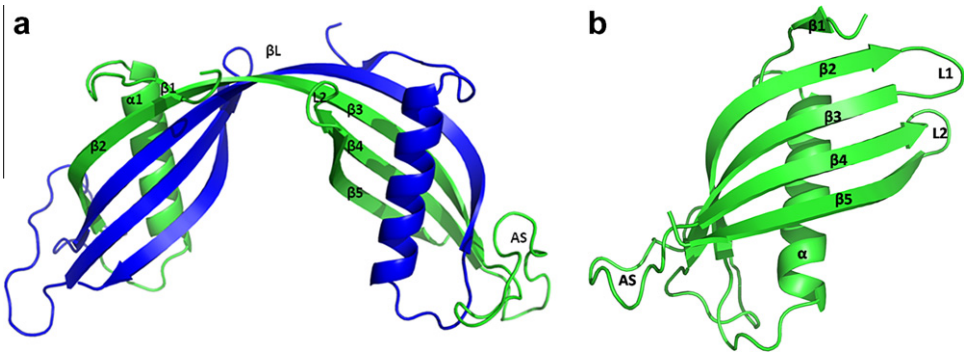

Fig. 1. Structure of the wild-type hCC folded as 3D domain-swapped dimer (a). Structure of hCC V57N folded as a monomer (b). This and all other structural illustrations were prepared using PyMol (DeLano, 2002).