Chapter 6

The functional integrity of the serpin domain of C1-Inhibitor depends on the unique N-terminal domain, as revealed by a pathological mutant

Ineke Bos, Yvonne Lubbers, Dorina Roem, Jan Pieter Abrahams, Erik Hack and Eric Eldering

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ABSTRACT

C1-Inhibitor (C1-Inh) is a serpin with a unique, non-conserved N-terminal domain of unknown function. Genetic deficiency of C1-Inh causes hereditary angioedema. A novel type of mutation (Δ3) in exon 3 of the C1-Inh gene, resulting in deletion of Asp55-Thr136 in this unique domain, was encountered in a hereditary angioedema pedigree. Because the domain is supposedly not essential for inhibitory activity, the unexpected loss-of-function of this deletion mutant was further investigated. The Δ3-mutant and three additional mutants starting at Pro76, Gly98 and Ser113, lacking increasing parts of the N-terminal domain, were produced recombinantly. C1-Inh* and C1-Inh** retained normal conformation and interaction kinetics with target proteases. In contrast, C1-Inh** and Δ3, which both lack the connection between the serpin and the non-serpin domain via two disulphide bridges, were completely non-functional due to a complex-like and multimeric conformation, as demonstrated by several criteria. The Δ3-mutant also circulated in multimeric form in plasma from affected family members.

The C1-Inh mutant reported here is unique in that deletion of an entire amino acid stretch from a domain not shared by other serpins leads to a loss-of-function. The deletion in the unique N-terminal domain results in a "multimerization phenotype" of C1-Inh, because of diminished stability of the central β-sheet. This phenotype, as well as the location of the disulphide bridges between the serpin and the non-serpin domain of C1-Inh, suggests that the function of the N-terminal region may be similar to one of the effects of heparin in antithrombin III maintenance of the metastable serpin conformation.
The autosomal dominant disease hereditary angioedema (HAE) is caused by functional deficiency of C1-Inhibitor (C1-Inh). C1-Inh is the major inhibitor of Cls and C1r of the classical pathway of complement, and also an inhibitor of the contact system proteases factor XIIa, kallikrein and factor XIa. A lack of this inhibitor may result in recurrent episodes of acute, local, circumscribed oedema of the skin or mucosa. The most serious and potentially life-threatening manifestation of the disease is laryngeal oedema. HAE is inherited as an autosomal dominant trait; affected individuals are heterozygous. Based on the relative levels of functional and antigenic C1-Inh, two types of HAE have traditionally been described. In type I (±85% of HAE patients) defective expression of one allele results in low antigenic and functional levels. In type II (±15% of HAE patients) levels of functional C1-Inh are low, but C1-Inh antigen-levels are normal because of the presence of a dysfunctional mutant protein. Although heterozygosity would suggest functional C1-Inh levels of around 50% in HAE, these levels are lower than 50%, typically 5-30%, which is ascribed to increased C1 activation and C1-Inh consumption.

However, the description of low levels of non-functional C1-Inh mutants in patients with type I HAE, has demonstrated that the distinction between types I and II HAE is not absolute. For example low levels of dysfunctional mutant protein may occur in cases where the mutation also leads to defective protein secretion. Many different mutations can lead to dysfunctional C1-Inh, as has recently been reviewed.

C1-Inh is a human plasma glycoprotein and a member of the SERine Protease Inhibitor (SERPIN) family to which antithrombin III (ATIII), plasminogen activator inhibitor 1 (PAI-1), and α1-antitrypsin (α1-AT) also belong. Serpins are suicide inhibitors that function as a set mousetrap by forming highly stable complexes with their cognate proteases. In its active form, a serpin has a metastable structure with a flexible reactive site loop that protrudes from the core of the molecule, the central β-sheet. The key residues P1-P1' form the reactive site, a pseudo-substrate for the target protease. Upon proteolytic attack on this bond the reactive site loop is pulled into the central β-sheet, inducing a dramatic conformational change that results in the formation of covalent complexes between inhibitor and protease. Recent crystallographic studies support the idea that during complex-formation the catalytic triad of the protease becomes distorted, which may explain the remarkable stability of the complex. Because mutations in the conserved flexible hinge region convert most inhibitory serpins into substrates it is currently believed that the velocity of this conformational change determines whether complex-formation and inhibition occur or whether the serpin is cleaved and the protease released. Indeed type II HAE may result from mutations in this hinge region of C1-Inh.

The conformational flexibility of serpins is also demonstrated by the existence of additional, biologically relevant conformations that are more stable. One of these is a latent, inactive form, adopted naturally by PAI-1 and ATIII, in which the uncleaved reactive site loop is inserted into β-sheet A, and the C-terminal peptide stalk and strand 1C are reoriented along the side of the molecule. In ATIII and α1-AT this latent state can be artificially induced, but artificial formation of latent C1-Inh has never succeeded. Secondly, non-covalent multimerization can occur upon denaturation, heating or proteolysis at certain
sites in the reactive site loop. Also several disease-associated mutants of C1-Inh, ATIII and α1-AT have this multimeric conformation, in some cases leading to accumulation of huge intracellular protein aggregates and loss of cellular function in diseases like emphysema (reviewed by Lomas and Carrell). Point-mutations causing spontaneous multimerization can be located in diverse regions: in or near the hinge, near a conserved hydrophobic pocket at the C-terminus, in helix B at a site predicted to play a role in the opening of β-sheet A, and near helix F which overlies β-sheet A. Conformational changes associated with latency or multimerization may result from similar structural causes, as demonstrated by ATIII-Rouen VI, in which a single point mutation results in the formation of latent monomers or multimers, depending on temperature.

Next to its canonical serpin domain, C1-Inh has a unique N-terminal domain of 116 amino acids. Two disulphide bridges linking Cys121 to Cys406 and Cys526 to Cys183, connect the N-terminal to the serpin domain. Reduction of these disulphide bonds in the intact serpin reduces its conformational stability and induces a latent conformation by auto-insertion of the reactive site loop into the central A β-sheet. The N-terminal domain is unique for C1-Inh, and its exact function is unknown. Its first 98 residues were shown not to be essential for complex formation. Here, we describe a novel type of mutation in the C1-Inh gene that sheds additional light on the function of this domain. The mutation, detected in a Dutch HAE pedigree, involves a deletion of 165 basepairs in exon 3 of the C1-Inh gene leading to loss of amino acids 62 to 116. Although located in the non-serpin domain, the deletion resulted in loss of functional C1-Inh. The unique features of this pathological C1-Inh mutant prompted investigation of the structural basis for the phenotype. Unexpectedly, the results revealed that the cysteine-containing fragment of the N-terminal domain of C1-Inh stabilises the serpin domain by tethering the central β-sheet via disulphide bridges. Thus, the reactive site loop is prevented from spontaneous insertion; reminiscent of one of the roles played by heparin in ATIII.

**Materials and Methods**

*Materials*

$^{35}$S-methionine and $^{14}$C-methylated protein markers were obtained from the Radiochemical Center (Amersham Biosciences). Biotin was from Pierce (Rockford, IL), and coupled to proteins according to the manufacturer’s instructions. Purified human plasma C1-Inh was a gift from Behringwerke (Marburg, Germany). Dithiotreitol and soybean trypsin inhibitor (SBTI) were purchased from Sigma Biochemicals (Steinheim, Germany). N-ethylmaleimide (NEM) was purchased from BDH Biochemicals (Poole, United Kingdom). Cls for kinetic assays was purchased from Calbiochem (La Jolla, CA, USA). Human Factor XIIa and kallikrein were purchased from Kordia (Leiden, the Netherlands). Biotinylated polyclonal rabbit antibodies against human C1-Inh, and the monoclonal antibodies (mAb) RII (against human C1-Inh), and Kok12 (specific for cleaved or complexed human
C1-Inh) have been described before 44,45. C1s for ELISAs was purified and biotinylated (C1s-BT) as described before 44. Streptavidin coupled to polymerised horseradish peroxidase (poly-HRP) was obtained from Sanquin (Dept. Immune Reagents, Amsterdam, the Netherlands). Streptavidin coupled to monomeric horseradish peroxidase was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden).

Patient DNA purification and plasma parameters
Blood samples were collected in siliconized vacutainer tubes (Becton Dickinson, Plymouth, UK). SBTI (100 μg/ml), benzamidine (10 mM), and EDTA (10 mM) were added to prevent in vitro activation of the complement and contact systems. Tubes were centrifuged at 1,300 x g for 10 min, and plasma was aliquoted and stored at -70°C until testing. Cells were collected and genomic DNA was purified according to the high-salt precipitation protocol 46. Affected members were investigated for C1-Inh and C4 as reported previously 47, or utilising ELISAs described below.

PCR, plasmid construction, and protein expression
PCR on genomic DNA (0.5 μg) was performed with primers (0.5 μM) flanking exon 3 of the C1-Inh gene (28 cycles of 30 sec 90°C, 60 sec 56°C, 90 sec 72°C). Forward primer: CTGACTATCCCTCATCTTC; reverse primer: TTAGTGGCTGCGACCTTAT; 5' ends correspond with nucleotides 1951 and 2947, respectively, of the published C1-Inh gene sequence 48,49. Wild type and mutant PCR products were purified from agarose gels, subcloned in a T-tailed vector (Promega) and two independent clones were sequenced (USB, Cleveland, OH). Subcloning of relevant fragments was done by digestion with BamHI and EcoNI and insertion into the corresponding sites in the C1-Inh expression plasmid 50. COS-1 cell transfection, metabolic labelling, immunoprecipitation and SDS-PAGE were done as described before 15,50. PCR and subcloning for production of the N-terminal deletion mutants in P. pastoris was performed as described. Primers were designed that started at amino acid 76, 98 and 115 of C1-Inh according to the numbering of Bock et al. 41, and enabled subcloning of the mutant gene in the plasmid for expression and secretion by P. pastoris. P. pastoris GS115 transformation and C1-Inh expression were performed as described before 51.

SDS-PAGE and Western Blotting
Electrophoresis was performed on Biorad Mini Protean II and Hoefer SE600 systems. Immunoprecipitation of plasma samples or 35S-methionine labelled COS media was done with mAbs coupled to Sepharose 4B (Pharmacia). Western blotting of HAE and normal plasma was performed by first adsorbing plasma samples (20 μl) onto mAb RII-Sepharose overnight, followed by 5 washes with PBS containing 0.1% (w/v) Tween 20 (PT), and dissociation of bound C1-Inh by boiling in non-reducing sample buffer. Samples were electrophoresed on 7.5% gels and transferred to nitro-cellulose. Blots were blocked with 0.4% (w/v) skimmed milk powder in PBS and incubated with rabbit polyclonal anti-C1-Inh antibodies (2 μg/ml) for 2 hrs, followed by a 1 hr incubation with peroxidase conjugated
horse polyclonal anti-rabbit antibodies (1:1000, Sanquin). Blots were developed with chemoluminescence reagents from Amersham.

Reduction and alkylation of C1-Inh
Plasma C1-Inh (1 mg/ml) was incubated for 30 minutes at room temperature with 100 mM DTT and subsequently with 250 mM NEM to irreversibly alkylate the free cysteines. A control sample was treated with 250 mM NEM only. This preparation was dialysed twice against a 100-fold excess of PBS.

ELISAs
C1-Inh antigen was detected with an ELISA modified from a radioimmunoassay as described earlier. Functional human C1-Inh was detected as described. Briefly, C1-Inh was bound to solid-phase mAb RII against human C1-Inh and detected with biotinylated C1s. Notably, this assay is an end-stage, and not a kinetic, assay that measures the number of functional C1-Inh molecules rather than the kinetics of the interaction between C1s and C1-Inh. Conformationally changed C1-Inh was measured in an ELISA-system with the mAb Kok12 as coating antibody. This mAb reacts with neo-epitopes exposed on complexed or cleaved C1-Inh. C1-Inh multimers were detected in an ELISA with mAb RII both as coating and as detecting antibody. PBS-Tween (0.1% w/v) was used as the buffer for all incubation steps in the ELISAs.

Kinetics of the interaction between C1-Inh and target proteases
The interaction between C1-Inh and C1s, coagulation factor XIIa or kallikrein was studied with the method of progress curves as described before. Experiments were performed twice in triplicate with similar results.

Heat stability
Culture supernatants containing normal or mutant C1-Inh were incubated for 2 hours at different temperatures, followed by centrifugation at 10,000 x g for 20 minutes. Antigen remaining in solution was measured in an ELISA for C1-Inh antigen.

Sucrose density gradient centrifugation
Plasma samples (200 µl) were loaded onto 5-25% (w/v) sucrose gradients in PBS, and centrifuged at 35,000 rpm at 4°C for 16 hrs. Fractions were tested in ELISAs for C1-Inh antigen or multimers. Plasma fractions were also tested for C1-Inh, IgG and IgM on an automated nephelometer (Behringwerke), as a reference for the molecular weight of C1-Inh species.
RESULTS

C1-inhibitor protein and DNA sequence of the patient.
The index patient had a typical history of HAE, and was initially classified as a type I HAE based on levels of functional and antigenic levels of C1-Inh (17 % both) and low C4 levels (30 mg/l). However, upon analysis by immune precipitation and immunoblotting, plasma from a member of the affected family appeared to contain low levels of an aberrant, low molecular mass form of C1-Inh of about 70 kDa, which was smaller than the intact and cleaved forms C1-Inh of 104 kDa and 97 kDa, respectively, which naturally occur in human plasma (see figure 1).
DNA sequencing of the pedigree, with primers flanking exon 3 of the C1-Inh gene, yielded an anomalous, specific PCR fragment. In addition to the normal fragment of 997 basepairs, a shorter fragment of approximately 830 basepairs was observed which was cloned and sequenced (figure 2a and b, GenBank™ AY291075). It appeared that a deletion of 165 basepairs within the coding sequence of exon 3 had occurred. As is virtually always the case in HAE, the index patient was heterozygous for this deletion (see figure 2a). Exon 3 of the C1-Inh gene codes for part of the signal sequence, the entire NH2-terminal domain, and part of the serpin domain of C1-Inh. The deleted part is peculiar in nucleotide (27% A, 44% C, 12% G and 16% T) sequence. Many A/C rich direct repeats of up to 11 nucleotides are present, which may have caused the deletion to occur. In addition, it contains Cys103 and Cys106, which connect the N-terminal domain to the serpin domain by forming disulphide bridges with Cys203 and Cys206. This deletion of 165 basepairs predicted a deletion of 55 amino acids of the N-terminal non-serpin domain including two cysteines involved in the formation of the two disulphide bridges of the molecules. This mutant is further indicated as the A3 mutant.

Figure 1. Low molecular weight C1-Inh is detected on SDS-PAGE in affected HAE patient from a novel Dutch pedigree. Normal (lane 2) and HAE plasma (lane 1) were absorbed onto RII-Sepharose and bound C1-Inh was subjected to non-reducing 7.5 % SDS-PAGE. After transfer to nitro-cellulose the blot was developed with polyclonal anti-C1-Inh antibodies.
Expression and activity of C1-Inh mutants

In first instance, it seemed unclear why the in-frame deletion identified in the patient should cause HAE, as it is located outside the serpin domain, and as earlier studies have indicated that the N-terminal domain is dispensable for inhibitory capacity. An explanation could be that the deletion severely impedes secretion, thereby explaining the type I form of HAE. Expression of the Δ3 mutant in COS cells indicated, however, that the mutant was in fact secreted, albeit at lower levels than WT C1-Inh, but unable to form complexes with C1s. This suggested a disturbed functional interaction as the basis for disease, which is mostly
associated with type II HAE. To assess in detail this impaired function of the N-terminal domain at a protein level, we decided to express and analyse several additional N-terminal deletion mutants, starting at amino acid 76, 98 and 115 (C1-Inh<sub>76</sub>, C1-Inh<sub>98</sub>, and C1-Inh<sub>115</sub> respectively, summarised in figure 2c). To obtain sufficient amounts of recombinant protein for a detailed analysis of the inhibitory and kinetic properties of the N-terminal deletion mutants, we produced WT, C1-Inh<sub>76</sub>, C1-Inh<sub>98</sub> and C1-Inh<sub>115</sub> in P. pastoris. In a previous study, we have shown that WT C1-Inh can be conveniently expressed in this system and is fully functionally active. The proteins were expressed at levels of 30, 35, 34 and 2 µg/ml, respectively. Supernatants of transfected cells or P. pastoris were analysed by ELISA for C1-Inh antigen and function. It appeared that the mutants A3 and C1-Inh<sub>115</sub> were completely non-functional towards C1s (figure 3), whereas C1-Inh<sub>76</sub> and C1-Inh<sub>98</sub> displayed normal activity. Normal functional activity of the latter two mutants was confirmed in the kinetic analysis showing comparable association (k<sub>on</sub>) and dissociation (k<sub>off</sub>) constants of these mutants with C1s, kallikrein, and factor XIIa as WT C1-Inh (Table I). Thus, the decreased function of the A3 C1-Inh mutant likely was related to the absence of the linker sequence between the serpin domain and the N-terminal domain.

![Figure 3](image)

**Figure 3.** C1-Inh<sub>115</sub> and A3 mutants do not form complexes with C1s. Culture supernatants containing C1-Inh<sub>76</sub>, C1-Inh<sub>98</sub>, C1-Inh<sub>115</sub>, A3 mutants or WT C1-Inh were absorbed onto solid-phase mAb RII, and incubated with biotinylated C1s. Functional activity was expressed as a percentage of activity in normal human plasma.

### Table I

Association (K<sub>on</sub>) and dissociation (K<sub>off</sub>) rate constants of C1-Inh with various target proteases as determined with progress curves.

<table>
<thead>
<tr>
<th>Protease</th>
<th>WT C1-Inh</th>
<th>C1-Inh&lt;sub&gt;76&lt;/sub&gt;</th>
<th>C1-Inh&lt;sub&gt;98&lt;/sub&gt;</th>
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<tr>
<td></td>
<td>K&lt;sub&gt;on&lt;/sub&gt;* (M&lt;sup&gt;-1&lt;/sup&gt;s&lt;sup&gt;1&lt;/sup&gt;)</td>
<td>K&lt;sub&gt;off&lt;/sub&gt;</td>
<td>K&lt;sub&gt;on&lt;/sub&gt;* (M&lt;sup&gt;-1&lt;/sup&gt;s&lt;sup&gt;1&lt;/sup&gt;)</td>
</tr>
<tr>
<td>C1s</td>
<td>6.3*10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>2.3*10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>6.8*10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>Kallikrein</td>
<td>8.2*10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>2.1*10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>5.9*10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>XIIa</td>
<td>5.7*10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>1.4*10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>5.2*10&lt;sup&gt;3&lt;/sup&gt;</td>
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Heat-stability and conformation of normal and mutant C1-Inh

Functional serpins, including C1-Inh, are in a metastable state, which can be monitored by thermal unfolding of the protein. Serpins in a native conformation will precipitate or severely multimerize at elevated temperatures with concomitant loss of detectable antigen. However, upon inactivation by insertion of the reactive site loop into the central β-sheet, a serpin will remain soluble. Although WT C1-Inh and the two N-terminal deletion mutants with intact disulphide bridges (C1-InhΔe and C1-InhΔs) were precipitated at 50-60°C, C1-InhΔm and Δ3 remained detectable up to 80 °C (figure 4a). This indicated that the latter two mutant proteins are in an intrinsically stable conformation, as opposed to the metastable conformation of the wild-type protein. This was confirmed by the increased reactivity of C1-InhΔm and Δ3 with mAb Kok-12 (which recognizes a neo-epitope exposed on complexed and inactivated C1-Inh) compared with WT, C1-InhΔe and C1-InhΔs C1-Inh. Serial dilutions of C1-Inh were tested in the Kok-12 ELISA and C1-InhΔm and Δ3 displayed a profound reactivity with this conformation-specific mAb (figure 4b). Notably, no reactivity of these two mutants with the mAb KII, specific for the cleaved form of C1-Inh, was observed (data not shown) which indicates that the mutants were not conformationally changed due to proteolytic cleavage of the reactive site loop.

Multimerization of mutant C1-Inh in vitro

Because in other C1-Inh mutants the intrinsic exposure of the Kok-12 epitope and an increase in heat-stability were associated with spontaneous formation of non-covalent multimers\textsuperscript{13,35}, WT C1-Inh or deletion mutants were analysed in an ELISA designed to detect C1-Inh multimers. As can be observed in figure 5, the mutants Δ3 and C1-InhΔm indeed appeared to form multimers in contrast with WT C1-Inh, C1-InhΔe and C1-InhΔs. Multimers of Δ3 could also be observed upon size fractionation on sucrose gradients (results not shown). Together with the reactivity with mAb Kok-12 and the increased heat stability, these results suggested that the deletion mutant has a conformation similar to several C1-Inh mutants described previously, which was ascribed to overinsertion of the reactive site loop and/or release of strand 1C, resulting in multimer formation\textsuperscript{13,35}.

Role of disulphide bridges in the conformation of C1-Inh

Because the deletion encompasses only Cys\textsubscript{10} and Cys\textsubscript{15}, the two remaining cysteines in the serpin domain might form a novel disulphide bond, which given the location of Cys\textsubscript{183} and Cys\textsubscript{205} would probably severely disturb the typical serpin conformation. To study the presence of disulphide bonds in the mutant protein, the normal and mutant proteins were immunoprecipitated with mAb RII and compared on reducing and non-reducing SDS-PAGE. In figure 6a it can be seen that the normal protein displayed a difference in apparent molecular mass upon reduction; under reducing conditions it migrated with an apparent molecular mass of 97 kDa, as has been observed before (e.g. refs \textsuperscript{16,37}). The deletion mutant migrated at identical molecular mass under both conditions, and no disulphide-linked dimers could be observed (figure 6a, lanes 2 and 4), strongly arguing against the presence of novel intra- or intermolecular disulphide-bonds.
C1-INH metastability depends on unique N-terminal domain

Figure 4. C1-Inh\textsubscript{115} and Δ3 C1-Inh display a stable conformation.

a. Heat-stability; WT, C1-Inh\textsubscript{115}, C1-Inh\textsubscript{98}, C1-Inh\textsubscript{115} and Δ3 C1-Inh were incubated for 2 hours at the indicated temperatures, centrifuged for 20 minutes at 10000 x g and the supernatant was analysed in the ELISA for C1-Inh antigen.
b. C1-Inh preparations were analysed in ELISA with Kok-12 as catching antibody. The detection of Kok-12 reactive C1-Inh was plotted against the C1-Inh concentration. Experiments were performed 3 times with similar results.

Figure 5. Δ3 mutant forms multimers \textit{in vitro}.

Presence of C1-Inh multimers in supernatants containing WT C1-Inh or C1-Inh mutants were analysed in a sandwich ELISA with mAb RII as both catching and detecting antibody. The detection of multimers is plotted against the C1-Inh concentration. The experiment was performed 3 times with similar results.
Another possibility is that the absence of disulphide bridges itself caused the observed multimerization phenotype. We indeed validated that permanent disruption of the disulphide bridges in normal C1-Inh by reduction and subsequent alkylation led to intrinsic conformational changes, as evidenced by formation of C1-Inh multimers. Shown in figure 6b is the extensive multimer formation at 37°C for reduced/alkylated C1-Inh, whereas treatment with only alkylation reagent did not induce this state.

Figure 6. Role of disulphide bonds in the conformation of C1-Inh
a. Absence of 2 cysteines does not lead to intra- or intermolecular disulphide bond formation. Normal (lanes 1 and 3) and mutant (lanes 2 and 4) C1-Inh from metabolically labelled transfection media were immunoprecipitated with mAb RII and analysed under non-reducing (lanes 1 and 2) or reducing (lanes 3 and 4) conditions on 10% SDS-PAGE.
b. Disruption of disulphide bonds in plasma C1-Inh causes multimerization. Control C1-Inh (lane 1 and 2) or reduced/alkylated C1-Inh (lane 3 and 4) was incubated for 2 hrs at 37°C (lane 1 and 3) or 60°C (lane 2 and 4) at a concentration of 1 mg/ml. Samples were analysed by 4-15% native PAGE.

Figure 7. Presence of C1-Inh multimers in vivo. Samples (200 μl) of normal plasma (NP, closed symbols) or mutant HAE (∆3, open symbols) plasma were layered onto 5 – 25% sucrose gradients. Fractions were analysed in C1-Inh antigen (circles) and multimer (triangles) ELISA. For C1-Inh antigen ELISA, NP fractions were diluted 1:100, D3 fractions diluted 1:50, while the dilution for the multimer ELISA was 1:25. The IgG-peak as determined by nephelometric analysis was in fraction 27.
Conformation of mutant C1-Inh in vivo

To confirm the properties of the C1-Inh mutants, observed in vitro, in the naturally occurring situation, normal plasma and HAE plasma containing the deletion mutant were analysed by size on sucrose gradients. To decrease the chance of activation and/or multimerization in vitro, for these experiments samples were used that had not been thawed previously. The fractions were tested in ELISAs for C1-Inh antigen and multimers (figure 7). Normal plasma displayed one peak in the C1-Inh antigen assay, and no multimers were detected by ELISA. The plasma of the patient showed, in addition to a peak at the normal position, one or more peaks at fractions corresponding to higher molecular mass. Furthermore, only the patient plasma yielded a response in the multimer ELISA, at fractions corresponding to high molecular mass C1-Inh. Thus, the deletion mutant of C1-Inh is also secreted in vivo, and circulates in a form that is, at least partially, multimeric.

Discussion

Previously, we have described single-residue mutations in C1-Inh from HAE patients, that cause a conformational change involving the reactive site loop and result in spontaneous multimer formation and/or dysfunction. Such point mutations, also observed in α1-AT and ATIII are distributed over various regions of the serpin domain of C1-Inh. The mutation described here is peculiar, because it is not a point mutation of a highly conserved residue but a deletion of 55 residues in a domain that is unique for C1-Inh among the serpins. To investigate the effect of this deletion we produced several recombinant mutants, illustrated in figure 2c. While deletion of the majority of this domain, i.e. up to 97 amino acid residues, had no effect on C1-Inh function, a deletion including the disulphide bridges of this part completely disrupted C1-Inh activity. Our observations may help to understand the conformational changes involved in C1-Inh function and the role of the N-terminal domain including the cysteines that connect the serpin and the unique non-serpin domains in C1-Inh.

The phenotype of the C1-Inh mutant of the patient was a multimerization phenotype with no inhibitory activity. The Δ3 mutant as well as C1-Inh both displayed strongly enhanced reactivity in a multimer ELISA with a homologous sandwich of mAb RII (figure 5) and in an ELISA with mAb Kok-12 that reacts with neo-epitopes exposed on complexed or proteolytically cleaved C1-Inh (figure 4b). In other studies we have shown that the epitope for mAb Kok-12 is also expressed on C1-Inh multimers. Notably, patient plasma contained circulating Δ3 multimers, as demonstrated with a sucrose gradient and a multimer ELISA (figure 7), ruling out that the observed multimers of the recombinant mutants were due to in vitro artifacts.

The affected members of the pedigree with the Δ3 C1-Inh mutant were described as type I HAE based on their low C1-Inh antigen levels. This seems surprising when the effect on C1-Inh is considered to yield a dysfunctional protein, typically found in type II HAE. We hypothesise that the decreased levels of C1-Inh antigen leading to the diagnosis type I
in stead of type II result from varying mechanisms including impaired secretion of the mutant protein. During recombinant expression of the C1-InhΔ and Δ3 mutants we observed consistently lower expression levels than for WT C1-Inh or the functional N-terminal deletion mutants. Metabolic labelling studies in COS-cells were attempted without success to demonstrate intracellular localisation, but multimers were detected in Western Blots with cell extracts (data not shown). This suggests intracellular multimerization of these mutants in vitro, as has been described for other C1-Inh variants of the multimerization phenotype. However, the dysfunctional protein was also detected, though at low levels, in the plasma of the patient (figure 1). Although the clearance of serpin multimers in vivo has not been studied, that of complexed or inactivated serpins is increased compared with that of native serpins. Because C1-Inh multimers expose similar epitopes as complexes, as is demonstrated by the reactivity of mAb Kok12, the clearance mechanism via the low density lipoprotein receptor-related protein might be similar. This has in fact been experimentally supported by studies of Chang et al., who demonstrated competition between α1-AT and ATIII complexes versus multimers for binding to the low-density-lipoprotein receptor-related protein (LRP). Hence, increased clearance of the dysfunctional mutant may have contributed to the apparent type I HAE phenotype.

The explanation for the observed multimerization phenotype can be the absence of the unique N-terminal domain and/or the absence of the disulphide linkage between the N-terminal and the serpin domain. In a previous study, complex formation of truncated variants lacking amino acid 1-98 displayed the capacity to form complexes with several target proteases that were stable for at least 30 minutes. To fully appreciate the consequences of the mutations, however, kinetic data are essential because complex formation does not necessarily imply full functional activity. Production of C1-InhΔ and C1-InhΔΔ in the P. pastoris system yielded sufficient amounts of mutants for such detailed kinetic studies. We have shown before that WT C1-Inh from P. pastoris has similar kinetic properties as plasma-derived C1-Inh. Association and dissociation constants of the C1-InhΔ and C1-InhΔΔ mutants were essentially the same as for WT C1-Inh (see Table I). This definitely ruled out an essential role for the first 98 amino acids in inhibitory capacity of C1-Inh. It can however be postulated that the presence of the remaining part of the N-terminal domain of the Δ3 mutant prevents correct folding or function of the serpin domain. For that reason we produced C1-InhΔΔΔ, which lacks the entire N-terminal domain, and consists solely of the serpin domain. This mutant appeared to have the same phenotype as the Δ3 mutant identified in the patient. This observation pointed to the absence of residues 99 to 115 as the explanation for the phenotype of the Δ3 mutant, rather than folding abnormalities induced by the remaining part of the N-terminal domain.

A feasible explanation for the observed inactivity is the disruption of the disulphide bridges between Cys185 and Cys46 in the serpin domain with respectively Cys183 and Cys181 in the non-serpin domain. This may have at least two different effects. First, the conformational change could result from a novel disulphide bond between the remaining cysteines in the serpin domain, but SDS-PAGE analysis did not reveal a difference in migration under reducing and non-reducing conditions (figure 6a). Because the effect of
reduction and alkylation of plasma C1-Inh was similar to the effect of deletion of the two N-terminal cysteines (figure 6b, our own unpublished observations and \(^2\,^4\), it is most likely that the absence of the disulphide linkage between the two domains had caused the inactivity of the Δ3 mutant, rather than the presence of an intra-serpin domain disulphide bond. This interpretation is entirely in line with earlier results demonstrating that disruption of the disulphide bridges in full length C1-Inh results in the formation of a latent, inactive conformation \(^9\). Our results demonstrating the crucial role of residues 99 to 115 underline the importance of the disulphide bonds in the stabilisation of the active conformation of C1-Inh. The crucial role of the cysteines for serpin metastability is surprising because these residues are not conserved among the serpins.

We have previously put forward a 3-dimensional model of the serpin domain of C1-Inh based on homology with the crystal structures of 4 native serpins \(^1\). Comparison of the positions of the disulphide bonds in this model (figure 8a) with the structure of antithrombin III (ATIII) complexed to a pentasaccharide reveals an interesting homology (figure 8b) \(^6\). Native ATIII has a flexible central β-sheet with the reactive site loop partly inserted. Binding of a pentasaccharide closes the central β-sheet, thereby stabilising the metastable conformation and expelling the reactive site loop, resulting in potentiation of ATIII. Moreover, it is known that the presence of the pentasaccharide inhibits spontaneous auto-insertion of the reactive site loop of ATIII \(^6\). The heparin-binding region on ATIII is located in the same region as Cys183 in C1-Inh \(^2\). This suggests that the N-terminal domain of C1-Inh linked via disulphide bridges to the serpin domain has a similar function as heparin for ATIII, which is stabilisation of the central β-sheet to maintain the metastable conformation. Notably, ATIII without heparin is less stable than C1-Inh and latent ATIII is observed in plasma whereas latent C1-Inh has never been observed neither \textit{in vivo} nor \textit{in vitro} \(^2\). Several other mutations leading to HAE support the importance of this region for the metastable conformation of C1-Inh. First, mutation of the residues Gly162→Glu/Arg and Thr96→Pro leads to type I HAE \(^2\,^6\). These residues are located between Cys163 and Cys206 and their mutation might also affect stability of the central β-sheet. Secondly, mutation of Cys181→Tyr leads to type I HAE \(^6\). The second cysteine in the serpin domain of C1-Inh (Cys206) is located at the same site as the disulphide bridge that links the short N-terminal domain of ATIII to the serpin domain (Cys21 to Cys35). This suggests that the terminal part of the unique N-terminal domain of C1-Inh is located at a similar position as the short N-terminal domain of ATIII. Taken together these data strongly suggest that the role of the disulphide bridges in C1-Inh is similar to that of heparin binding for ATIII. However, whereas the activity of ATIII is functionally regulated by heparin at these sites, C1-Inh’s active conformation would be constitutively primed by the geometrical constraints imposed by the disulphide bridges in the context of residues 99 to 115 of its N-terminal domain. The inhibitory activity of C1-Inh, enhanced by heparin and other highly negatively charged polymers \(^6\,^6\) must therefore be potentiated through a different mechanism than that of ATIII.
Figure 8. Structure of C1-Inh compared to that of ATIII-pentasaccharide complex.

a. Homology model of C1-Inh based on the crystal structure of 4 different active serpins. The N-terminal Thr\(^{116}\) and the two cysteines in the serpin domain are indicated as ball-and-stick and labelled.

b. Crystal structure of ATIII bound to a pentasaccharide (1AZX \(^{61}\) from the Protein Database (http://www.rcsb.org/pdb/), viewed in WebLabViewLite (http://www.msi.com)). Phe\(_{121}\) and Cys\(_{96}\) of ATIII, at the same location as Cys\(_{183}\) and Cys\(_{406}\) of C1-Inh are indicated as ball-and-stick and labelled. The pentasaccharide is also indicated as ball-and-stick and labelled.
CONCLUSIONS

A novel pathological C1-Inh mutant with a deletion of 55 amino acids in the unique N-terminal domain of C1-Inh was identified. Studies of a recombinant variant of this mutant and N-terminal deletion mutants starting at amino acids 76, 98 and 115 indicated that C1-InhA3 and Δ3 C1-Inh displayed a conformational change leading to multimerization, whereas C1-InhΔ5 and C1-InhΔ8 displayed a wild-type like conformation. The Δ3 C1-Inh and the C1-InhA3 mutants were dysfunctional because the absence of the disulphide bridges between the serpin and the unique N-terminal domain led to destabilisation of the metastable conformation of the serpin domain. This stabilising function of the N-terminal domain of C1-Inh resembles that of heparin for ATIII. Thus, a broader perspective is offered for understanding aberrant protein folding and/or multimerization by intermolecular β-sheet interactions, leading to pathologies which include HAE, emphysema, Alzheimer and the prion encephalopathies.

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