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Structural and Functional Aspects of C1-Inhibitor

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Abstract

C1-Inh is a serpin that inhibits serine proteases from the complement and the coagulation pathway. C1-Inh consists of a serpin domain and a unique N-terminal domain and is heavily glycosylated. Non-functional mutants of C1-Inh can give insight into the inhibitory mechanism of C1-Inh. This review describes a novel 3D model of C1-Inh, based on a newly developed homology modelling method. This model gives insight into a possible potentiation mechanism of C1-Inh and based on this model the essential residues for efficient inhibition by C1-Inh are discussed.

Introduction

C1-Inhibitor

C1-Inhibitor is the major inhibitor of C1s and C1r, two serine proteases that together with C1q constitute the C1 complex of the classical pathway of complement (1–3). C1-Inh also inhibits the contact system proteases XIa, XIIa, and kallikrein and is therefore an inhibitor of several pathways of inflammation (4–8). C1-Inh can also interact with the so-called mannan binding lectin (MBL)-associated serine proteases or MASPs: C2, C3, or C4 consumption by MASP-1 or -2 in serum can be inhibited by addition of C1-Inh (9). However, the relevance of this interaction *in vivo* is unclear since no kinetic data of the interaction of C1-Inh with MASPs are available.

C1-Inh purified from pooled human plasma has been used since the 1970's as a treatment for patients with hereditary angioedema (HAE), a disease often caused by a heterozygous deficiency of C1-Inh. Application of C1-Inh in inflammatory conditions like sepsis, acute myocardial infarction and vascular leakage syndrome seems a promising treatment (reviewed by CALIEZI et al., (10) and discussed in other parts of this issue). C1-Inh application in inflammatory disease is, however, hampered because of the high doses required. It has been shown that heparin and other glycosaminoglycans can enhance inhibitory capacity of C1-Inh (11, 12). Dextran sulphate seems to be the most effective glycosami-

Abbreviations: C1-Inh = C1 esterase inhibitor; serpin = serine protease inhibitor

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noglycan, which can enhance C1-Inh function 130-fold *in vitro* and 60-fold in plasma. However, this effect of dextran sulphate is limited in vivo, probably due to dissociation of the DXS-C1-Inh complex and the subsequent fast clearance of dextran sulphate (13).

Mature C1-Inh is a protein of 478 amino acids, and is heavily glycosylated (approximately 30% by weight) (14). Though on SDS-PAGE gels it migrates with an apparent MW of 104 kDa, the calculated MW is lower being 76 kDa (15, 16). The molecule contains 13 glycosylation sites (14). C1-Inh consists of an N-terminal domain of 113 amino acids, and a serpin domain of 365 amino acids. The function of the N-terminal domain is discussed below. Two disulphide bridges linking cysteine 101 to cysteine 406 and cysteine 108 to cysteine 183, connect the N-terminal and the C-terminal domain. The structure of the serpin domain is homologous to that of other serpins, and is essential for the inhibitory capacity of the molecule. Compared to other plasma serpins, C1-Inh is a poor inhibitor although the inhibitory capacity of C1-Inh can be enhanced by glycosaminoglycans. Here, a novel 3D model of C1-Inh is proposed, based on the crystal structure of 4 inhibitory serpins. This may provide the basis to investigate the mechanism of potentiation of C1-Inh, and to compare this mechanism with that of the serpins antithrombin III, heparin cofactor II, and protein C inhibitor.

Serpins

C1-Inh is a member of the serine protease inhibitor, or serpin, superfamily of proteins. This family constitutes a class of proteins highly conserved throughout evolution, and characterised by a common structure. The main function of many serpins is to regulate the activity of serine proteases. However, some serpins may act to regulate the activity of other types of proteases as well (17), whereas other serpins, for example ovalbumin, may not function as an inhibitor at all. α1-Antitrypsin is considered as the archetype of serpins. Structural data of this and other serpins have provided a molecular explanation for the inhibitory mechanism of serpins. Serpins are suicide inhibitors, which function as a mousetrap. They are made up of three β-sheets and nine α-helices. In addition, they have a reactive site loop, which is a flexible peptide loop positioned outside the central β-sheet, and which is free to interact with target proteases. Key residues of this loop are the so-called P1 and P1′-residues which are located next to each other and constitute a bait for target-proteases. Upon cleavage of this bond by a target protease, the reactive site loop is pulled into the central β-sheet (Fig. 1), which causes a dramatic conformational change. As a result, the P1-residue of the reactive centre, while still covalently linked to the active site serine of the protease, is moved to the opposite pole of the molecule (18). The driving force for this movement is provided by the affinity-based insertion of the reactive site loop into the central β-sheet, from P15 to P1. Once moved to the opposite pole of the serpin, the catalytic site of the protease is significantly disturbed and no longer able to complete the hydrolysis process necessary to disrupt the bond between its active site serine and the P1 residue. This inability results from a disruption of the catalytic triad of the active site due to the pulling force of the reactive site loop inserted into the β-sheet, which probably directly results from the limited length of this loop. Hence, the protease becomes trapped by and attached to the serpin. This probably not only makes the protease inactive, but also more vulnerable to proteolytic attack (19). The complex of serpin and protease is rapidly cleared from the circulation. In case of C1-Inh the half-lives of clearance of complexes with various target proteases from plasma vary from 20 to 47 minutes (20, 21).

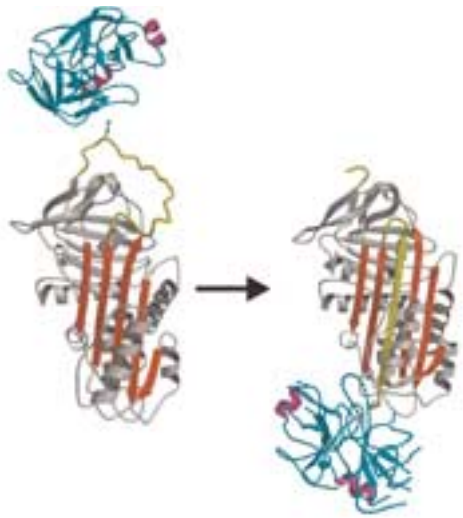

Figure 1. Formation of the complex of serpin and protease. Ribbon depictions of native α1-antitrypsin with trypsin aligned above in the docking orientation. (left), and of the complex shopwing the 71 Å shift of the P1 methionine of α1-antitrypsin, with full insertion of the cleaved reactive-centre-loop into the central β-sheet. Regions of disordered structure in the complexed trypsin are shown as interrupted coils projected from the native structure of trypsin. Red; α1-antitrypsin the central β-sheet; yellow, reactivesite loop, green ball and stick, P1 Met; cyan, trypsin (with helices in magenta for orientation); red ball and stick, active serine 195. Reprinted by permission from Nature (407: 923–926) copyright (2000) Macmillan Magazines Ltd.

Conformational changes resembling those occurring during complex formation with a protease, may occur in a serpin under some other conditions as well. First, they may be induced upon cleavage of a peptidyl bond of the reactive centre by a so-called non-target protease. This peptidyl bond is not necessarily the one between the residues at the P1 and P1′-positions. For example, neutrophil elastase can cleave the reactive site loop of C1-Inhibitor at P2–P3 and P4–P5. Following such a cleavage, the reactive site loop inserts into the central β-sheet, but now without a protease attached to it. A comparable conformational change also occurs when the serpin changes from the active into a so-called latent conformation. This conformational change results from a partial insertion of the uncleaved reactive site loop into the central β-sheet. Conversion from the active to the cleaved or latent state results in increased thermostability and loss of inhibitory activity (See for further details WHISSTOCK et al. (22)). It is believed that latent and cleaved serpins can act as signals for a range of physiological responses (23).

Development of a 3D model for C1-Inh

General approach

Though the crystal structure of C1-Inh has not been elucidated yet, that of several other intact serpins including antithrombin-III, α1-antitrypsin, ovalbumin, and serpin K have

become available last few years. Amino acid identity of C1-Inh with each of these serpins is about 25–30%. Hence, the reliability of a 3D model based on the structure of one of these serpins is low. On the other hand, though the overall identity is 25–30%, the homology between C1-Inh and each of these serpins may be much higher when individual stretches of up to 10 amino acid residues are considered. Hence, a model obtained by combining the structural data of several serpins, may be more reliable.

We designed a chimeric molecule with a carbon backbone consisting of fragments of the 4 crystal structures of the intact serpins antithrombin-III, α1-antitrypsin, ovalbumin, and serpin K. Alignment of local sequences of C1-Inh with those of antithrombin-III, α1-antitrypsin, ovalbumin and serpin K, was performed "manually", instead of using one of the web-based servers that serve as a black box lacking a check on sequence alignment when several templates are used. Stretches of 4 or 10 amino acids of C1-Inh were compared with equal stretches of the 4 serpins. A mainchain Cα backbone was constructed by connecting the 3D structures with the highest homology. A more detailed description of the modelling method is given below.

3D protein-modelling based on local alignment

3D co-ordinates of the protein atoms and the secondary structure of several serpins were obtained from the PDB (http://www.rcsb.org/pdb/) (24). The co-ordinates of the files 1QLP, 2ANT, 1OVA and 1SEK were used for, α1-antitrypsin, antithrombin-III, ovalbumin, and serpin K, respectively (25–28).

Sequence alignment for C1-Inhibitor with 6 serpins (α1-antitrypsin, antithrombin-III, ovalbumin, serpin K, α1-antichymotrypsin, and plasminogen activator inhibitor-1) was performed with ClustalX 1.8, Clustal W 1.8 and ClustalW 1.75 (29, 30). 3D alignment of the serpins was performed in O on a Silicon graphics computer, the root means square deviation of their structures was calculated in O, a mainchain Cα backbone was constructed by connecting the 3D structures with the highest homology using the program "O" (31). Side-chains were incorporated with Whatif (32). Sequence alignment of 2 sequences was performed with the program Blast on the World Wide Web (33). The multiple sequence alignment generated by ClustalX 1.8 was adapted at minor points according to the alignment by ClustalW 1.75. This was used as a basis for local alignment. No programs are available for local alignment of short stretches of 4 or 10 amino acids. All commonly used sequence alignment programs are apt to compare long stretches of amino acids. We developed a sequence alignment program in which one stretch of 4 or 10 amino acids was compared to all possible stretches of equal length in the closely surrounding area of the 4 serpins. All these stretches were given a score based on the matrix OBlosum62. Sequences with the highest scores and at least a 3 amino acid overlap were used as a basis for the chimeric molecule. This resulted in the sequence alignment based on the 3D structure, which is shown in figure 2.

In the 3 dimensional chimeric molecule the carbon backbone was constructed by visual rebuilding performed in the program O. When more than one serpin had a very high homology score a decision was made based on the 3D structure. At certain sites where the amino acids did not exactly link up, rigid bodies of carbon atom backbones were moved or rebuild using the "Lego auto main chain" command in O; visual rebuilding. This database scans a 3D database for fragments with similar carbon α backbones. The remaining insertions and deletions were inserted/deleted in the PDB file of the best matching ser-

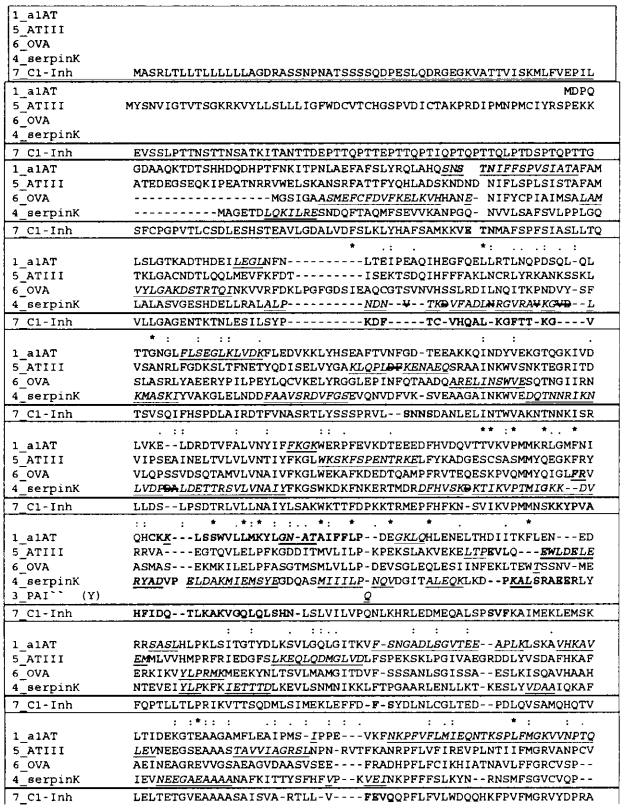

Figure 2. Multiple sequence alignment of C1-Inh with 4 other serpins. The alignment was performed as described in Materials and Methods. Underlined sequences form the template for the chimeric structure

pin and the backbone was rebuilt as mentioned above. After a first round of visual rebuilding the chimeric sequence was aligned with C1-Inh. At one specific site around amino acid 306–316 (KKYPVAHFIDQ in Figure 2), where the sequences did not align, the backbone was revised in order to obtain optimal alignment.

The carbon backbone was visually scanned for possible atomic clashes, and rebuild at clashing sites after which atomic clashes were calculated. The side chains of C1-Inh residues were built in with the program Whatif. Checks for bumping side-chains were performed visually. The standard minimisation procedures were performed in Whatif with the commands DEBALL and Refine until the Z-scores of the structure did not further improve.

The 3D-model of C1-Inh

The 3D model of the serpin domain of C1-Inh is depicted in Figure 3 and the coordinates are available in the protein data base under accession number 1M6Q(RCSB ID:

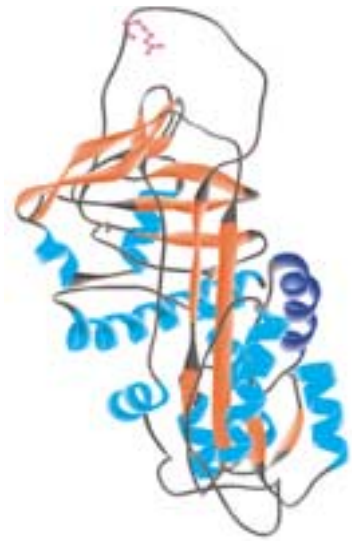

Figure 3. 3D model of C1-Inh. Carbon backbone with secondary structure (α-helices in light blue, β-strands in red). Dark blue, D-helix; magenta ball-and-stick; P1, Arg 444

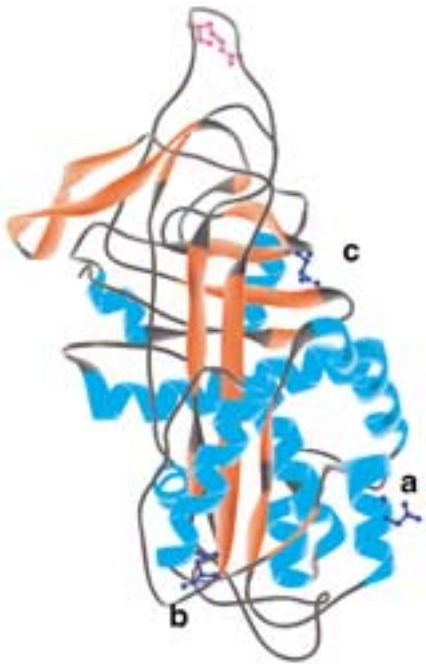

Figure 4. Glycosylation sites in the serpin domain of C1-Inh. Magenta ball-and-stick; P1 arg 444. Dark blue ball-and-stick; the side-chains of the glycosylated residues asn 216 (a), asn 231 (b) and asn 330 (c)

RCSB016664) (http://www.rcsb.org/pdb/). The r.m.s. fit of the model compared to the 4 template serpins was between 1.425 and 1.661 Å. The model starts at amino acid 116 of C1-Inh (numbering according to Bock et al. (14)), and hence lacks the aminoterminal domain. We did not incorporate this domain in the model since its sequence is unique and lacks homology with any protein sequence in the Protein DataBase (PDB), based on a Psi-blast search (34). Compared to the other serpins C1-Inh has a short or no D-helix and a reactive site loop that is on average two amino acids shorter. This is illustrated in figure 2 and 3. The reactive site is formed by arg 444 (35) (14), as is shown in figure 3. C1-Inh contains thirteen glycosylation sites. Ten of these sites are located in the N-terminal domain. Three sites are located in the serpin domain at amino acids 216, 231 and 330 (14). These are depicted in figure 4. The surface charge of C1-Inh according to the 3D-model was calculated with the program WebLabViewerLite (www.msi.com) (data not shown). Analysis of the surface charge of C1-Inh results in three potential heparin-binding regions. Region 1 is located close to P1 and the secondary C1s binding site that has been postulated by He et al. (36, 37). Region 2 is located close to the secondary C1s binding site though somewhat further from P1. Region 3 is located around helix D.

Accuracy of the 3D-model

Due to the lack of structural data, the accuracy of the model cannot be determined. To have some indication about this accuracy, we applied the same modelling method to protein C Inhibitor (PCI) (data not shown), and looked for mutagenesis data of this serpin regarding its interaction with glycosaminoglycans (GAG), in the literature. For PCI, four possible GAG binding regions were defined by analysis of the surface charge of the 3D model of PCI. Indeed, one of these regions contains all the residues that have been defined by site-directed mutagenesis to be responsible for potentiation of PCI by heparin (38–40). Thus, our modelling method at least may be used to define possible GAG binding regions.

For comparison, we also produced a 3D model of C1-Inh based on α1-antitrypsin in Whatif and we obtained a model from Swissmodel based on the same 4 serpins as we used (32, 41, 42). These models differ from each other mainly at sites where the sequence alignments do not agree, supporting that sequence alignment is the major problem of homology modelling under these conditions. Hence, we suggest that local alignment probably results in a more reliable model.

Carbohydrate groups

The function of the carbohydrate groups in C1-Inh is unknown. Deglycosylation of C1-Inh with N-glycanase, O-glycanase or both has no major effect on the functional activity of C1-Inh. C1-Inh is still able to form complexes with C1s (43). Addition of a glycosylation site around lys 251 had no effect on complex formation with C1s, kallikrein or XIIa (44).

Removal of the sialic acid groups from C1-Inh enhances the clearance of C1-Inh by the liver in a rabbit model (45), probably by binding to hepatic asialoglycoprotein receptors. Subsequent removal of penultimate galactose residues results in clearance rates near

normal. In the same study the inhibition of C4 activation by C1s was studied with desialated C1-Inh. This inhibition was equal to inhibition by normal C1-Inh. Together these results suggest that glycosylation of C1-Inh contributes to its clearance from plasma, but has no major effect on its functional activity. However, regarding the latter it should be noted that kinetic studies of the influence of the different carbohydrate groups on the interaction of C1-Inh with target proteases have not been performed. Hence, it cannot be ruled out definitely that the carbohydrate groups of C1-Inh may contribute to its functional activity. In figure 4 the glycosylation sites of C1-Inh are depicted. It is well possible that one or several of the carbohydrate groups play a role in the stabilisation of the central β-sheet, especially asn 231. Because the rate of inhibition is critically dependent on the stability of this β-sheet, glycosylation might play a subtle role in the protease trapping. Asn 330 may play a role in stability of the reactive site loop and enhance the association of C1-Inh with proteases, though because of its distance from the P1-residue, this effect probably will not be profound. On the other hand, asn 330 is located not far from the secondary binding site for C1s on C1-Inh, which has been defined by HE et al. (37), but still a 20.21 Å ± 2.56 Å distance between asn 330 and phe 455 is present in this model. Although it is not known to impair complex formation of C1-Inh and C1s, deglycosylation still may influence the binding kinetics.

The N-terminal domain of C1-Inh

The function of the N-terminal domain of C1-Inhibitor is unclear. As indicated above, it does not share significant homology with any known protein, and hence the amino acid sequence of this domain does not give a clue regarding its function. Truncated variants of the molecule have been prepared in vitro. Two of these variants, starting at amino acid 98 or 76, respectively, were able to form complexes with C1s, C1r, factor bXIIa and kallikrein (46). In addition, they effectively inhibited C1 activity in haemolytic assays. These variants had equal thermal denaturation profiles compared to wild-type C1-Inh, indicating their serpin moiety had the native configuration. Two disulphide bridges are formed between the N-terminal and the C-terminal domain, which disulphide bridges are intact in the truncated variants described above. Reduction of these bonds by dithiothreitol causes a conformational change and insertion of the reactive site loop into the central β-sheet (47). Thus, the disulphide bridges probably prevent transition of the native configuration of C1-Inh into a latent conformation. Moreover, the importance of the disulphide bridges is supported by the mutation of cys 183 to tyr, found in a in an angioedema patient (48).

The N-terminal domain may also play a role in the clearance of C1-Inh. Most of the glycosylation sites are located in the N-terminal domain. Studies have shown that altering glycosylation influences the clearance (45). Truncated variants of C1-Inh have not been studied yet regarding their interaction with glycosaminoglycans. Hence, it is not known whether this domain contributes to the potentiation of the inhibitory function of C1-Inh.

Mechanism of potentiation of C1-Inh

The mechanism of potentiation of antithrombin III by heparin has been well defined. Heparin binds specifically through a core pentasaccharide to antithrombin III resulting in a conformational change resulting in a transition of the main sheet of the molecule from a partially six-stranded to a five-stranded form, while pushing out the reactive site loop to give a more exposed orientation (49). It can be concluded from our 3D-model of C1-Inh that this mechanism does not explain the potentiation of C1-Inh by glycosaminoglycans. The reactive site loop of C1-Inh is 5 amino acids shorter than that of antithrombin III, which virtually rules out that it can form a small side loop interacting with the central sheet. The major heparin-binding site of antithrombin III is the D-helix, which helix in C1-Inh according to our model comprises the amino acid range 183–196. However the D-helix in our model is shorter than the D-helix of any other serpin, and this region shows very low homology with other serpins. The residues lys 189 and lys 194 are postulated as possible heparin binding candidates but whether this region of the protein really comprises an α-helical structure is uncertain. Heparin cofactor II also binds heparin through the D-helix while protein C-inhibitor binds heparin primarily via its H-helix (39, 40, 50). The H-helix of C1-Inh comprises amino acid residues 358–366. According to our 3D-model none of the amino acid residues of this helix contribute to the postulated glycosaminoglycan binding sites, though Lys 368, which is just outside the H-helix, is one of the residues postulated to contribute to one of the positively charged regions of C1-Inh. Hence, the H-helix of C1-Inh is unlikely to affect the potentiation mechanism of C1-Inh.

Potentiation of the inhibitory capacity of C1-Inh by glycosaminoglycans differs for each of the target proteases. The inhibition of C1s or factor XIa is potentiated, while that of factor XIIa or kallikrein is not (51), as is summarized in table 1. Although our 3D-model may give a clue about the binding sites for glycosaminoglycans on C1-Inh, it does not provide an explanation regarding these dissimilar effects of various target proteases.

It can be concluded that based on structural considerations, the mechanism of potentiation of C1-Inh by glycosaminoglycans differs from that of ATIII, and probably also from that of protein C inhibitor. However, whether these statements indeed are correct should be proven in further experiments.

Table 1. Potentiation of C1-Inh by glycosaminoglycans. Effect of potentiation on different target proteases

Target protease	Potentiation by clycosaminoglycans yes/no
C1s	yes
XIa	yes
XIIa	no
Kallikrein	no

Protease specificity

Protease specificity of serpins is in particular determined by the nature of the amino acid residue at the P1 position of the reactive site loop. In C1-Inh this residue is arginine at position 444, predicting specificity for trypsin-like proteases. Indeed all known target proteases of C1-Inh belong to the trypsin-branch of serine-proteases. However, the residue at the P2 position, in C1-Inh alanine at position 443, also influences target protease recognition. Mutation of ala 443 to val diminishes the inhibitory activity of C1-Inh towards C1s and C1r, though this mutant protein can inhibit trypsin, and has a normal interaction with XIIa and kallikrein (52). Mutation of ala 443 to asp, which has a bulky and charged side chain, results in defective inhibition of C1r, and XIIa and impaired inhibition of C1s and kallikrein (53). This suggests that the mechanism of binding of C1-Inh to the various target proteases is different, which is supported by the fact that potentiation with heparin-like molecules does not occur for all target proteases. Wild-type C1-Inh is cleaved by human neutrophil elastase. Mutation at the P3 and P5 residue results in resistance to this catalytic inactivation while inhibitory capacity is not affected (54). Protease specificity is mainly related to the conformation of the reactive site loop. Hence, the mutations that lead to an impaired interaction with certain proteases, probably influence the accessibility of the reactive site. Most striking is the ability of the ala 443-val mutation to inhibit trypsin. Wild-type C1-Inh hardly inhibits trypsin, rather it is mainly inactivated by this protease. Apparently, trypsin efficiently cleaves the peptidyl bond between arg at P1 and thr at P1′, and has completed this cleavage before the reactive site loop is completely inserted in the central sheet. However in the ala 443-val mutant hydrolysis of the ester bond is less efficient, leaving sufficient time for the trapping mechanism of the inhibitor. In other words, the insertion of the reactive site loop of the mutant is completed before the bond between the active site serine of trypsin and arg at P1 is hydrolysed. Consequently, the catalytic triad of trypsin becomes disturbed while the active site serine is still bound to arg 444, and the complex between trypsin and C1-Inh is stabilised.

Inhibitory capacity

The inhibitory capacity of C1-Inh is determined by its abilities to bind a protease and to trap the protease according to the mechanism typical for serpins. The former requires adequate exposure of the reactive site loop and the association constant (k_{on}) reflects the rate of protease binding. The latter requires a fast insertion of the reactive site loop into the central β-sheet that should be completed before the bond between the active site serine of the protease and arg 444 is hydrolysed. The ability to keep the protease trapped is reflected by the dissociation constant (k_{off}). In theory, a molecule with a very high k_{on} and a very high k_{off} is not an inhibitor, but rather a suitable substrate. In general, the types of mutations found in C1-Inh can be divided in three classes. Class I comprises mutations that lead to altered exposure of the active site. The mutants described in the section "Protease specificity" probably all belong to this class. Class II comprises mutations leading to disturbed insertion of the reactive site loop; the serpin will serve as a substrate in stead of an inhibitor. Class III contains mutations with spontaneous insertion of the reactive site loop in the same or another molecule, leading to formation of a latent molecule

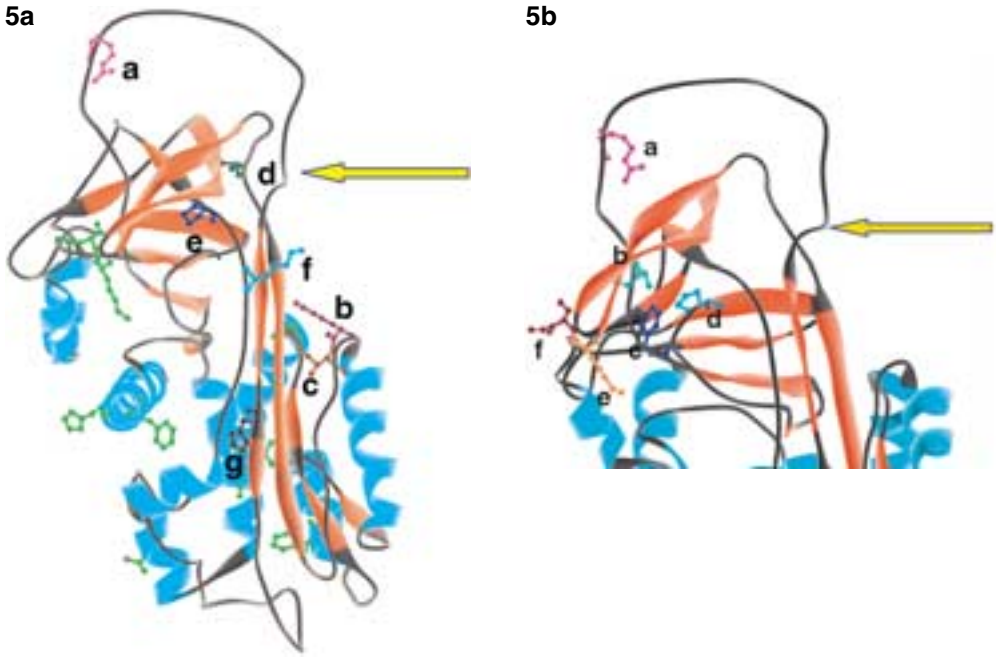

Figure 5. C1-Inh inhibitory mechanism. Ribbon diagram with localization of residues involved in the inhibitory capacity of C1-Inh. Yellow arrow; hinge region. Marked residues are shown in ball-and-stick Fig. 5a; side view of C1-Inh. Magenta; P1 arg 444 (a): different colors; indicating mutated residues that probably result in class II deficient inhibition; purple: Lys 251 (b); brown: Ile 252 (c), dark green: Thr 279 (d) dark blue: Pro 377 (e); light blue: Lys 380 (f) black: His 421 (g). Green; mutations resulting in HAE but unexplained structural consequences. Fig. 5b; side view of the central β-sheet, sheet C, and the hinge region of C1-Inh with mutated residues that result in class III deficient inhibition; Magenta; P1 arg 444 (a): green: val 451 (b); dark blue: phe 455 (c); light blue: pro 476 (d); orange: lys 294 (e); brown: asn 295 (f)

or polymers. Several mutations in the hinge region of C1-Inh (P14, P12, and P10) convert C1-Inh into a substrate for its target proteases (Class II), probably because the docking of the reactive site loop of these mutants into the central sheet is too slow, which impairs the trapping of the protease. The hinge region is indicated in Figure 5a and is located at the N-terminal region of the reactive site loop. Davis has reviewed the mutations in this region of C1-Inh in 1997 (55). A mutation at P10 from ala to thr results in blocked protease recognition and polymerisation of the molecule (class III) (56). Mutations in the C-terminal region of the reactive site loop at the conserved residues val 451, phe 455 and pro 476 also lead to multimerization. This is probably due to deterioration of anchoring of the reactive site loop resulting in overinsertion of the loop into the central β-sheet, which subsequently leads to multimerization (Class III) (57, 58). All these residues are located in β-sheet C, between the central β-sheet (A) and the reactive site loop (Fig. 5b). Several other mutations in the C-terminal region of C1-Inh (Phe 457 Leu and Met 470 Lys) have been reported in HAE patients. Whether these mutations result in less production of C1-Inh or in a dysfunction of the molecule is, however, not

known (59). It is supposed that mutants leu 459 pro and pro 467 arg are incorrectly folded, while mutants at Phe 455, Pro 476 and Val 451 multimerize (58). All residues are located in β-sheet B, behind the central β-sheet, but it is hard to conclude what the consequence of the mutations at phe 457 and met 470 is.

Deletion of lys 251 leads to multimerization of C1-Inh and conversion to a substrate (44). Deletion of this residue results in a new glycosylation site. However the dysfunction of this mutant could not be attributed to the presence of an additional carbohydrate group, since production of this mutant in the presence of tunicamycin did not restore function. Deletion of lys 251 is likely to disrupt the structure of the central β-sheet, because lys 251 is located in a loop overlying the central β-sheet (Fig. 5a). This mutant formed no complexes with C1s, C1r or kallikrein and inefficiently formed complexes with factor XIIa. Each of the proteases induced partial cleavage on the mutant inhibitor, indicating insufficiency of the trapping mechanism (Class II).

Recently Bowen has described four newly found mutations (59), and reviewed several mutations not reviewed before (48, 60, 61). This comprises mutations in the following residues: Phe 127 Val, His 133 Arg, Lys 155 Pro, Gly 162 Glu/Arg, Thr 169 Pro, Cys 183 Tyr, Val 196 Asp, Ser 224 Arg, Ile 252 Val, Thr 279 Lys/I, del lys294 and Asn 295, Pro 377 Ser/Ala, Lys 380 Ile, and His 421 Arg. We do not provide a detailed description of these mutants but based on our model, as depicted in fig 5a, some speculations can be made. The original articles provide a more detailed description and also some speculations about the structural effect of these mutations. Lys 380 is located in the central β-sheet and it is likely that this mutant protein serves as a substrate due to insufficient trapping, like for example the mutant with deletion of lys 251 (Fig. 5a). Pro 377 and His 421 are located in the central β-sheet, Ile 252 is located next to Lys 251. Like described for Lys 251 it is likely that protease trapping is inefficient, due to disruption of the central β-sheet, resulting in cleavage of the serpin (Class II). Thr 279 is located next to the hinge region, and mutations are also likely to disturb efficient trapping of the protease and conversion to a substrate (Class II). Lys 294 and 295 are located in β-sheet C and might provide anchoring of the reactive site loop. Mutation probably leads to multimerisation or formation of latent C1-Inh (Class III). The other mutations are located in the helical regions behind the central β-sheet and the function of this region is not known.

Interaction with C1s has been postulated to involve another site distinct from the P1-P1′ residues of the reactive site loop of C1-Inh, which site may be essential for formation of a stable enzyme-inhibitor complex. A synthetic peptide corresponding to the sequence of the residues 448 to 459, the distal hinge region, was shown to inhibit the complex formation. This peptide bound to C1s, and C1s preincubated with this peptide cleaved C1-Inh rather then forming stable complexes (36). As a control, a peptide identical to the sequence of the reactive site of C1-Inh also bound to C1s but did not enhance cleavage of C1-Inh. Thus, the site on C1-Inh near to the distal hinge region was claimed to interact with C1s and to be necessary for adequate entrapment of this protease by C1-Inh. Gln 452, gln 453 and phe 455 were claimed to be the critical residues for this secondary C1s binding site (37). Some of the autoantibodies found in patients with acquired angioedema may bind to this region (62). The importance of this secondary binding site for C1s has, however, been challenged by a study showing that mutation of the residues 452 and 453 to alanine had no effect on the association rates of C1-Inh with C1s and did not impair complex formation between C1-Inh and C1s, C1r, kallikrein or XIIa (63). However, in this study the quantification of C1-Inh for kinetic analysis was

merely based on a determination of active C1-Inh. It is unclear whether this reflects the amount of total C1-Inh. When only a minority of C1-Inh is active, this was not detected but might be due to this specific mutation.

It has been shown that many different residues can be involved in the inhibitory capacity of C1-Inh. Some residues are important for flexible insertion of the reactive site loop upon protease binding, some may be involved in an additional C1s binding site, whereas others are important to maintain the exposure of the reactive site loop.

Conclusions

C1-Inhibitor belongs to the class of serpins and consists of a serpin domain and a unique N-terminal domain. The function of the N-terminal domain is unclear. Recombinant C1-Inh without the N-terminal domain is able to bind to target proteases but the kinetics of this interaction are not known. The effect of the thirteen glycosylation sites in C1-Inh on protease inhibition is also unknown. Although the binding to target proteases is possible without carbohydrate groups, the kinetics of this interaction might be influenced by glycosylation. A novel homology modelling method has been used in this study to develop a new model of C1-Inh, based on the 3D structure of 4 different serpins. Based on the surface charge of this model a possible glycosaminoglycan binding site has been predicted, but the exact binding site remains to be determined by site-directed mutagenesis. It can however be concluded that the mechanism of potentiation is different from the potentiation of antithrombin III by heparin. Mutations in C1-Inh can provide insight into residues that are crucial to serpin function. The reactive site (P1; arg444) does not only determine protease specificity, but also by the adjacent residue (P2; ala 443). An overview of mutations that have been described by other investigators in this 3D model illustrates residues that are crucial for the inhibitory mechanism of C1-Inh. Residues around the active site are crucial to maintain correct protease specificity. Residues around the hinge region as well as regions in or in contact with the central β-sheet are crucial to maintain inhibitory capacity and prevent conversion to a substrate, while anchoring of the reactive site loop is essential to prevent loop overinsertion or multimerisation. When essential residues in these regions are mutated, the functionality of the mousetrap is disturbed, either by inefficient or by preliminary trapping.

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