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REVIEW ARTICLE

TOWARDS UNDERSTANDING OF HELIX B BASED CONFORMATIONAL DISEASES IN SERPIN

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Abstract: Serine protease inhibitors (serpins) are a unique family of protease inhibitors that are prone to polymer formation due to their metastable nature and a complex inhibition mechanism that involves large scale conformational change. Helix B is in the shutter region near the strand 2A and strand 3A of β-sheet A, where reactive centre loop inserts during the serpin inhibition mechanism. Helix B region in serpins is a mutation hotspot for naturally occurring variants that result in pathological conditions due to polymerization. Helix B residues are completely buried in the native state and loop inserted latent state but not in the inhibitory loop inserted cleaved conformation. Native to cleaved transition during inhibition forms a large cavity in the shutter region, which invariably is the largest cavity in most serpins in native state. In a recent paper we had for the first time hypothesized that exposure of helix B at the N-terminal end is important for smooth insertion of the reactive center loop during serpin inhibition mechanism. It is therefore possible that natural variant that induces conformational deformation of helix B probably alter the cavity size which increases the rate of loop-sheet interaction between the monomers resulting in increased polymerization.

Keywords: Serpins; Protein polymerization; α1-Antitrypsin; Shutter domain; Conformational diseases

Serine Protease Inhibitors and Conformational Diseases

Minor mutations can modify the folding of the proteins to cause a decreased conformational stability that consequently results in a number of diseases (Potempa *et al.*, 1994). The term 'conformational disease' covers numerous heterogeneous disorders which include dementias, thrombosis, Alzheimer's disease, Parkinson's disease, Prion spongiform encephalopathies, Mad Cow disease and many other diseases (Carrell and Lomas, 1997; Nar *et al.*, 2000). Serine Protease inhibitors (serpins) are a super-family of proteins that controls the proteinases; they are prone to conformational diseases due to its inhibition mechanism and are associated with diseases like emphysema, cirrhosis, angioedema, familial dementia, chronic obstructive bronchitis and thrombosis (Silverman *et al.*, 2001). Serpins are the largest and most widely distributed family of proteinase inhibitors. The family is characterized by more than 30% sequence homology and a common framework of tertiary structure (Silvermann *et al.*, 2001). More than 700 different serpins have now been identified in diverse organisms including viruses, mammals, plants, insects and most recently in bacteria (Carrell and Owen, 1985; Stein and Carrell, 1995; Huntington and Carrell, 2001). In human plasma, serpins represent 2% of the total protein of which 70% is α_1-antitrypsin (Carrell and Lomas, 1997). Serpins like α_1-antitrypsin, α-antichymotrypsin, C1-inhibitors, antithrombin and plasminogen activator inhibitor-1, play absolutely critical roles in the control of proteinases involved in the inflammatory, complement, coagulation and fibrinolytic pathways respectively (Elliot *et al.*, 1996).

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A Precarious Inhibition Mechanism that Leads to Polymerization

Crystal structures have demonstrated that serpins are composed of three β-sheets (A-C), seven-nine α helices (A-I) and an exposed mobile reactive centre loop, that presents a peptide sequence as a pseudo-substrate for the target proteinase (Figure 1) (Lomas *et al.*, 2005). The critical amino acids within this loop are P1-P1′ residues, which acts as a 'bait' for the target enzyme (Cabrita and Bottomley, 2004). After docking the enzyme cleaves the P1-P1′ peptide bond of the serpin (Getting, 2002) and proteinase is activated by a mouse trap action that swing it 70 Å(1 Å = 10^{-10} m) from the upper to the lower pole of protein in association with insertion of a reactive centre loop as an extra strand (s4A) in β-sheet A (Sivasothy *et al.*, 2000). The reactive loop/β-sheet A interaction of serpin is crucial for their role as effective antiproteinase but also render them liable to undergo conformational transition that causes disease (James *et al.*, 1999). It has been shown that a similar A-sheet transition from a five-stranded to a hyperstable six-stranded form can occur in the serpin even if the reactive loop is intact. Thus, the uncleaved reactive loop of serpin can be inserted into its own β-sheet A, to form what is called latent structure. Alternatively, the opening of β-sheet A of one molecule can allow the entry of uncleaved reactive loop of another molecule to form a dimer, which then extend into long chain of polymer (Corral *et al.*, 2005).

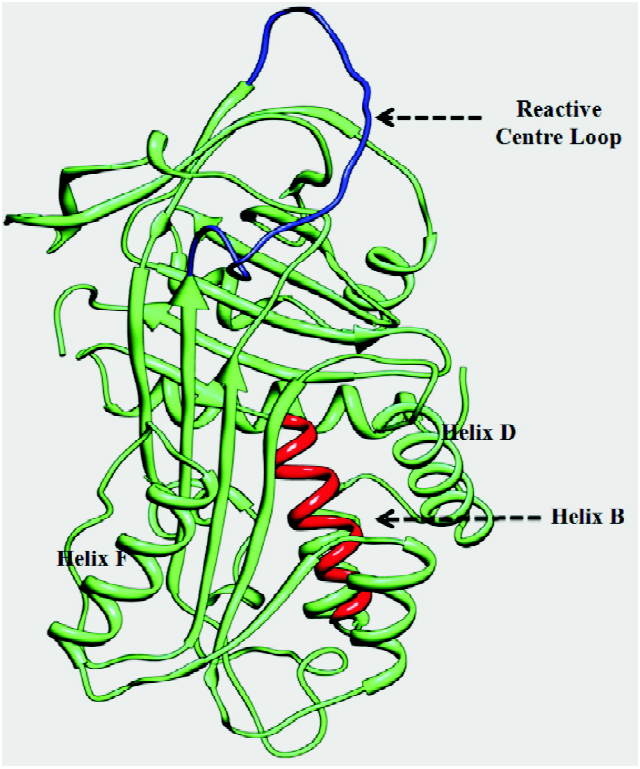

Figure 1: **Localization of helix B (in red color) in the serpin structure. Helix B is an important part of shutter area which is prone to misfolding and polymerization**

Polymerization of Serpins

The elegant mechanism of proteinase inhibition by serine proteinase inhibitors is sometimes subverted by point mutations resulting in the formation of ordered polymers that are retained within the endoplasmic reticulum of secretory cells (Lomas *et al.*, 2005). Serpin polymerization forms the basis for the great majority of all serpin related disorders or serpinopathies. This polymerization can be attributed to the inherent flexibility and metastability of the native serpin fold. Mutation in serpins can lead directly to functional defects or polymer formation that can compromise the specific function and leads to protein aggregation and accumulation of polymers that can cause cell toxicity and death. Thirteen different missense mutations of Antithrombin have been identified that leads to oligomer formation or conversion to an inactive "latent form (Getting, 2002). Similar types of natural variants have also been characterized in α-$_1$-antitrypsin (Lomas *et al.*, 2005), neuroserpin (Miranda and Lomas, 2006) and heparin cofactor II (Corral *et al.*, 2005). In native serpins, the polymerization can be induced in vitro by incubation at temperatures higher than 60°C and at mild guanidine hydrochloride concentration (Bottomley and Chang, 1997). These polymers are formed through two related mechanisms. One is due to point mutations that destabilize the native state of the serpin, such that it adopts a partially unfolded conformation with a high propensity to polymerize (Cabrita *et al.*, 2002). The second mechanism is that the mutation disturbs the normal folding pathway of the serpin, slowing the process down, leading to the accumulation of a partially folded conformation which subsequently polymerizes (Cabrita *et al.*, 2002).

Prevention of Polymer Formation

There is substantial evidence that polymers of α_1-antitrypsin and indeed all other serpins are formed by an aberrant linkage between the reactive centre loop of one molecule and β-sheet A of another (Cabrita and Bottomley, 2004). Understanding the structure and functional basis of polymerization is important to help the rational design of drugs that may lead to the development of novel therapeutic strategies to prevent diseases. It is known that polymerization of serpins occurs as a result of insertion of reaction centre loop of one molecule between strands 3 and 5 of another molecule (as s4A). It is also known that short peptides competitively bind to and block this s4A position of serpins to prevent polymerization (Zhou *et al.*, 2004). Also a hydrophobic pocket in α_1 antitrypsin has been identified; this pocket is present in the native protein but is filled as β-sheet A accepts an exogenous reactive loop peptide during polymerization. Agent that can bind to this pocket will predictably stabilize β-sheet A and inhibit polymerization (Elliot *et al.*, 2000). Chemical chaperones have shown some initial promise in reversing the polymerization in diseases like cystic fibrosis, Parkinson's, Alzheimer's and Gaucher's disease (Gelman and Kopito, 2002). Use of chemical chaperones like glycerol and trimethylamine N-oxide has shown promise in reducing the native states conversion to polymerogenic form in antitrypsin and neuroserpins (Devlin *et al.*, 2001; Zhou *et al.*, 2003).

Significance of Helix B in Serpins

Shutter region constitutes F-helix, B-helix, strands s3A and s5A of β-sheet which play an important role in stability and function of serpins (Dunstone *et al.*, 2000; Devlin and Bottomley, 2005; Gooptu and Lomas, 2009**).** Helix B is located at the upper portion of the shutter region where RCL inserts as s4A as shown in Figure 1. Clustal W analysis of some representative serpin clearly indicates there is an absolute conservation of proline and isoleucine residues in helix B among all native serpins, and corresponding residue numbers are given in Table 1 (Krem and Di Cera, 2003).. Helix B region in serpins is with several point mutations which result in pathological conditions due to polymerization. Helix B interacts with the β-sheet A amino acids which are conserved among various serpins as shown in table. Helix B mutations in α-1antichymotrypsin (Leu55Pro) and α1-antitrypsin (Phe51Leu, Ser53Phe and Val55Pro) can cause lung (emphysema) and liver diseases (cirrhosis). Protein C-inhibitor (Ser52Phe and Ser54Leu) and antithrombin (Pro80Ser/Thr, Thr85Met/Lys, Cys95Arg and Leu99Phe) have mutations which can result in angioedema and thrombosis, similarly in neuroserpin Ser49Pro and Ser52Arg are linked to hereditary disorder called familial encephalopathy with neuroserpin inclusion bodies (FENIB) (Lomas *et al.*, 1992; Lomas and Carrell, 2002; Crowther, 2002; Lomas *et al.*, 2005). It has been shown that network of residues dynamically coupled with each other, especially inside cavities and local and global stability of these regions mediate inhibitory activity (Sengupta *et al.*, 2009). Hence different mutations within the serpin can allow aberrant conformational transitions characterized by beta strand exchange between the reactive loop of one molecule and β-sheet A of another (Gooptu and Lomas, 2009).

Table 1
ClustalW Analysis of the helix B Region of Serpin

Helix B alignment	*Serpin*	*Residue numbers*
DNIF LSPLSIST	Antithrombin	74-85
TNIF FSPVSIAT	Antitrypsin	48-58
TNIV FSPLSISA	Antichymotrypsin	69-80
ENIL FSPLSIAL	Neuroserpin	44-55
DNIF IAPVGIST	HCFII	130-141
QNIF FSPVSISM	C1-Inhibitor	44-55

HCFII is heparin cofactor II,
Highly conserved regions are marked in orange

Experimental Studies of Helix B Region

The effect of point mutations on the rates of polymerization has been studied experimentally in antitrypsin. In this study six mutants were prepared and their propensity for polymerization was studied. These variants were naturally occurring like Z, S, I and M alpha antitrypsin as well as mutations induced into a recombinant like Phe51Leu, Glu354Gln, Glu354Ser. Results showed an increased rate of polymer formation with a

reduced melting temperature (Dafforn *et al.*, 2004). A previous study of Phe51Leu variant of α-1 antitrypsin showed increased stability and full activation as an inhibitor of elastases (Kwon *et al.*, 1994; Elliot *et al.*, 1996). A genetic and biochemical study of antithrombin mutations in shutter region showed the formation of disulfide linked dimers which leads to severe venous thrombosis. Antigen and anti factor Xa activity from plasma of patients carrying mutations like Pro80Ser and Gly424Arg in the antithrombin gene was analyzed and showed aggregation (Corral *et al.*, 2004). Five novel point mutations in antithrombin like M32T, M89K, L146H, Q159X and L409P has been identified in Japanese patients with type I deficiency. Out of these point mutations M89L is the one which lies in helix B region (Keiko *et al.*, 2003).

Possible Role of Helix B

Helix B analysis for residue burial and cavity was undertaken to understand its role in serpin structure function. A structural overlap and an accessible surface area analysis showed the deformation of strand 6B and exposure of helix B at N-terminal end in cleaved conformation but not in the native and latent conformation of various inhibitory serpins (Singh and Jairajpuri, 2011). Cavity analysis showed that helix B residues were part of the largest cavity in most of the serpins in the native state which increase in size during the transformation to cleaved and latent states (Singh *et al.*, 2012). These data for the first time showed the importance of strand 6B deformation and exposure of helix B in smooth insertion of the reactive center loop during serpin inhibition and indicate that helix B exposure due to variants may increase its polymer propensity (Khan *et al.*, 2011). Large scale conformation change involved in serpin inhibition mechanism also increases its tendency to polymerize. The results clearly indicated that the deeply burial of helix B residues and its presence inside cavities can cause the global destabilization in the variants of serpins which may result in increased conformational flexibility and might explain its increased polymer propensity. Helix B exposure facilitates smooth insertion of RCL as strand4A and transition of proteinase to the opposite end of the sheet A, which is mediated by stability changes, hydrogen bond switch and large cavity formation. Conformational changes in helix B variants that allow insertion of RCL of another molecule might trigger the large cavity formation resulting in trapping of inserted RCL in an irreversible complex that leads to polymerization.

Serine Protease Inhibitors as a Model System to Understand Conformational Diseases

Serpin can exist in various conformational states with variable cofactor binding ability. Several serpin have broad protease specificity, for example antithrombin can inhibit factor Xa, thrombin and factor IXa with very high efficiency. Also serpin like heparin cofactor II, antithrombin and protein C inhibitor can all inhibit thrombin (Aracos *et al.*, 2001). There are co-factor induced and non-cofactor induced serpins which differ in their function although they have similar structure. For example, antithrombin, heparin cofactor-II, protein C inhibitor, plasminogen activator inhibitor-1, protease nexin-1 and kallistatin can bind to a variety of ligands and cofactors like heparin, dermatan sulfate, vitronectin and N-acetyl glucosamine (Patson *et al.*, 2004). Serpin also include some members that act as inhibitors of other types of proteinases, while others are non-inhibitors. The diversity of a closely related family based on functional role, cofactor binding ability, and inhibition mechanism makes serpin an ideal system to gain residue level insight into various processes associated with serpin structure function and diseases. A collective analysis of serpin polymer inducing natural variant of serpins has given insight into the importance of residue burial linked shift in the conformational stability that increases its polymer propensity. A study of serpin protease complexes identified critical evolutionary conserved residues in exosites that determines its protease specificity. Specific knowledge of structural variations that gives rise to conformational defects in a super family like serpin can be extended to understanding protein conformational diseases in general.

Conclusion

Several polymer causing natural variants of serpins are known with no known effective treatments. One in 2000 individuals in Europe

gets affected with Z type α_1-antitrypsin variant (E342K) which causes cirrhosis and emphysema but has no cure. Mutant variants of serpins readily form long chain polymers that gives rise to clinical conditions that result from either protein overload and cell death or plasma deficiency. Identifying the mechanism of serpin dysfunction is linked to finding appropriate strategy for its cure. The serpin conformation, its complex inhibition mechanism, its metastable native state, variable cofactor binding abilities and availability of a large database of mutants makes it a potent model to study conformational diseases.

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Abbreviations

Serpin, serine protease inhibitors; RCL, reactive center loop; ATIII, antithrombin; HCFII, heparin cofactor II; PAI, plasminogen activator inhibitor, PC1, protein C1-Inhibitor,

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