Review Article

Structure-function Relationships in Serpins: Current Concepts and Controversies

Ann Gils, Paul J. Declerck

From the Laboratory for Pharmaceutical Biology and Phytopharmacology, Faculty of Pharmaceutical Sciences, Katholieke Universiteit Leuven, Belgium

Introduction

Almost two decades ago, a new superfamily of proteins was proposed, based on the similarity between the primary structure of ovalbumin, α1-antitrypsin (also known as α1-proteinase inhibitor) and antithrombin III (1). Even though the acronym “serpins” (serine proteinase inhibitors) was given to these proteins (2), it became soon apparent that a variety of non-inhibitory serpins [e.g. ovalbumin (3), maspin (4), pigment epithelium-derived factor (5)] as well as serpins that inhibit cysteine proteinases [e.g. interleukin-1 converting enzyme inhibitor (6), cathepsin L inhibitor (7)] belong to this superfamily. Serpins represent about 10% of the total protein in plasma. From these serpins, α1-proteinase inhibitor represents 70%. A number of serpins play a critical role in the regulation of important physiological processes such as blood coagulation (e.g. antithrombin III), fibrinolysis (e.g. plasminogen activator inhibitor 1, PAI-1), complement activation (e.g. C1-inhibitor), and inflammation (e.g. α1-antichymotrypsin) (8) (Table 1). The serpins comprise more than 40 proteins identified from viruses, plants, insects and animals but not from prokaryotes (9). All serpins consist of about 400 residues with molecular masses in the range of 38 to 70 kDa (dependent on the degree of glycosylation) and an overall amino acid homology of approximately 35% (10). Huber and Carrell (10) reported that the conserved residues are localized either internal or in niches on the surface. All serpins have the same highly ordered tertiary structure consisting of 3 β-pleated sheets A, B and C, α-helices A through I and a reactive site loop containing residues P16 to P10 (10, 11) (Fig. 1). The reactive site, designated P1P1’ and comprising the bait peptide bond, is located within this loop structure situated 30-40 amino acids from the carboxy-terminal end. Using the nomenclature of Schechter and Berger (13), the residues N-terminal to the scissile bond are designated the P-residues (P16 up to P1) whereas the residues to the C-terminal end are designated the P’-residues (P1’ up to P10’) (Table 2).

Even though almost all serpins contain Cys residues, only few of them (α1-antiplasmin, C1-inhibitor and antithrombin III) harbor disulfide bridges (10). For α1-antiplasmin it was recently shown that abolishing the disulfide bridge does not influence the thermodynamic stability, the inhibitory activity and the clearance by the receptors (14). Even though reduction of one of the three antithrombin III disulfide bonds does not affect the rate of thrombin inhibition as such, it does abolish the heparin-induced acceleration of the thrombin-antithrombin III interaction (15). These findings suggest that under certain conditions disulfide bridges may play a role in the functional properties of a serpin.

Structural Properties

The first X-ray structure of a serpin derivative was resolved in 1984 (16). This structure of human α1-antitrypsin cleaved at the P1P1’ (Met-Ser) site revealed that the new N- and C-termini were separated by 70 Å (16) (Fig. 2a). The new C-terminal residue (P1) is located at the end of the newly formed β-strand s4A within a six-stranded antiparallel β-sheet whereas the new N-terminal end (P1’) forms the new β-strand s1C. This conformational change yields a thermodynamically stable conformation and provides a structural explanation for the irreversibility of serpin inhibition. The structures of other cleaved inhibitory serpins, such as α1-antichymotrypsin (17), equine leucocyte elastase inhibitor (18) and bovine antithrombin III (19) confirmed the insertion of the P1-P16 portion of the reactive site loop into β-sheet A. In contrast, the structure of cleaved ovalbumin (plakalbumin), revealed that this cleaved non-inhibitory serpin has not undergone such a conformational change upon cleavage (20) (Fig. 2b). In this structure, the residues P14 to P7 are in a random coiled conformation without formation of β strand s4A and resulting in a P7 to P1’ distance of only 27 Å. The failure of reactive site loop insertion was attributed to the presence of two “large” (i.e. valine) residues at positions P11 and P12 (21, 22), the presence of a phosphorylated Ser at position P9, the presence of a charged residue (Arg) at position P14 (cfr. Table 2), the shielding of Leu at position 193 from solvent, or the removal of the hexapeptide at the cleavage site (20). Consequently, the existence of a direct link between a lack of inhibitory activity and a lack of insertion of the reactive site loop into β-sheet A was made (23). However, the crystal structure of a cleaved, non-inhibitory substrate variant of PAI-1 [PAI-1-Ala(335, P12)–Pro] revealed the insertion of the P16 to P3 residues in β-sheet A (24) (Fig. 2c) in spite of the lack of inhibitory activity. Biochemical studies on a variety of other substrate-type mutants of serpins have further confirmed that insertion upon cleavage is generally occurring in these non-inhibitory variants (25-27).

In an intact inhibitory α1-antichymotrypsin mutant (28) and in active α1-antitrypsin (29), the reactive site loop is a distorted helix without insertion. The reactive site loop of an active mutant of α1-antitrypsin (30) revealed a canonical conformation ready for insertion (Fig. 3a) whereas the reactive site loop in antithrombin III is partially inserted up to P14 into β-sheet A between strand s5A and s3A (31, 32). From the...
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Table 1: Inhibitory and non-inhibitory serpins

<table>
<thead>
<tr>
<th>Inhibitory serpins</th>
<th>Target Protease</th>
<th>Function</th>
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<tbody>
<tr>
<td>α1-antitrypsin</td>
<td>Neutrophil elastase</td>
<td>Proteolysis</td>
</tr>
<tr>
<td>Antithrombin III</td>
<td>Thrombin, factor Xa</td>
<td>Clotting</td>
</tr>
<tr>
<td>α2-antichymotrypsin</td>
<td>Cathepsin G</td>
<td>Inflammation</td>
</tr>
<tr>
<td>PAI-1</td>
<td>t-PA, u-PA</td>
<td>Fibrinolysis</td>
</tr>
<tr>
<td>α2-antiplasmin</td>
<td>Plasmin</td>
<td>Fibrinolysis</td>
</tr>
<tr>
<td>C1-inhibitor</td>
<td>C1s, C1t, kallikrein</td>
<td>Complement activationfactor XIIa</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Non-inhibitory serpins</th>
<th>Function</th>
</tr>
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<tbody>
<tr>
<td>Corticosteroid binding globulin</td>
<td>none</td>
</tr>
<tr>
<td>Thymosin binding globulin</td>
<td>none</td>
</tr>
<tr>
<td>Angiotensinogen</td>
<td>none</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>none</td>
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* limited list, for complete list of serpins see (8)

Currently available structural data it is not clear whether or not this pre-insertion is a prerequisite for proteinase binding.

The crystal structure of the intact non-inhibitory serpin ovalbumin, revealed an α-helical reactive site loop indicating that the α-helix has to unfold prior to or during interaction with the target proteinase since helical residues are not accessible to cleavage (21, 33) (Fig. 3b). Because of its intrinsic lability, no crystal structure of active PAI-1 is available yet. However, the crystal structure of the latent conformation is solved (34). In this latent structure (Fig. 3c), strand s4A is inserted into β-sheet A from the “hinge” at P15 to P4 whereas the residues from P1’ to P10’ are located on the surface of the molecule. As a consequence, the bait region (P1P1’) and secondary binding sites are not accessible to the active site of the serine proteinases. In addition, a latent form of antithrombin III revealed a similar insertion (31).

**Target Specificity**

It is generally accepted that the P1 residue is the major determinant of the proteinase specificity of serpins (2). The active site serine hydroxylation group of the proteinases is critical for generating a high affinity interaction with the serpins (35). More than 40-60% of the binding free energy of the serpin-proteinase interaction is lost when the active site serine of the proteinase is altered. Changes at the P1 residue of the serpins through natural mutation, in vitro site-directed mutagenesis or species variation, result in an altered target specificity. Replacement of the P1 residue (Arg) in antithrombin III with Cys (36), Pro (37) or His (38-40) resulted in a reduction or loss of inhibitory activity. The Pittsburgh variant of α1-proteinase inhibitor in which Met at position P1 is replaced by Arg, converted α1-proteinase inhibitor to an inhibitor of thrombin ($k_2 = 3.1 \times 10^2 M^{-1}s^{-1}$ vs $k_2 = 48 M^{-1}s^{-1}$ for wt), of factor XIIa ($k_2 = 5.1 \times 10^3 M^{-1}s^{-1}$ vs $k_2 = 6.6 \times 10^3 M^{-1}s^{-1}$ for wt), of plasma kallikrein ($k_2 = 8.9 \times 10^4 M^{-1}s^{-1}$ vs $k_2 = 4.2 M^{-1}s^{-1}$ for wt), and of factor XIIa ($k_2 = 2.5 \times 10^4 M^{-1}s^{-1}$ vs non-inhibitory for wt) (41-44). Heparin cofactor II (Leu at P1) inhibits thrombin ($k_2 = 1 \times 10^3 M^{-1}s^{-1}$) but substitution with Arg results in a 100-fold increase of the inhibition rate constant ($k_2 = 1 \times 10^5 M^{-1}s^{-1}$) (45). Replacement of the P1 residue (Leu) in α1-antichymotrypsin by Met, Arg or Trp resulted in a target specificity for elastase ($k_2 = 4 \times 10^3 M^{-1}s^{-1}$), trypsin ($k_2 = 5.4 \times 10^3 M^{-1}s^{-1}$) and chymase ($k_2 = 1.5 \times 10^4 M^{-1}s^{-1}$), respectively (46-48) whereas altering the P1 residue (Arg) of C1-inhibitor into Glu or His revealed non-inhibitory variants (49, 50). Mutagenesis of the P1 residue (Arg) of protein C inhibitor to Met generated a mutant which reacts slower ($k_2 = 3.7 \times 10^2 M^{-1}s^{-1}$ vs $k_2 = 1.7 \times 10^3 M^{-1}s^{-1}$) with activated protein C (51). In PAI-1, a basic residue (Lys or Arg) is required at the P1 position for inhibition of urokinase-type plasminogen activator (u-PA) (52) whereas the presence of neutral or hydrophobic residues at this position does not affect tissue-type plasminogen activator (t-PA) inhibition properties (53). Inhibition of u-PA still occurs when P1’ is replaced by any amino acid except proline (52).

Substituting the residues at positions P2 and P3 of PAI-1 revealed the possibility for target specific inhibition, with t-PA being more tolerant than u-PA for structural diversity at the P2 and P3 positions (54). Moreover, replacing the residue at position P6 or P10 in PAI-1 by a proline results in a preferential inhibition of t-PA or u-PA without affecting the inhibition rate constants, respectively, whereas replacing the residue at position P18 in PAI-1 by a proline results in a PAI-1 variant exhibiting exclusively u-PA inhibitory properties (55). The underlying molecular cause for the influence of the residues remote of the P3P3’ region, on the target specificity of a serpin remains unclear.
It should be stressed that in the majority of these reports, mutations did often not result in an absolute target specificity but merely in a relative target specificity as only changes in the inhibition rate constants were observed. It is of interest to note that the inhibition rate constant at which serpins inhibit their target proteinases is also influenced by cofactors. One of these cofactors is heparin, a highly sulphated linear polysaccharide that accelerates the inhibition rate constant of the heparin activatable serpins, such as antithrombin III, PAI-1, protease nexin I, protein C inhibitor, heparin cofactor II, with their target protease. The most pronounced increase (4000-fold) in inhibition rate constant is observed when heparin binds to antithrombin III (56, 57). The heparin-induced acceleration of the antithrombin III-thrombin interaction is explained by a template mechanism (56, 57) whereas the antithrombin III-factor Xa interaction is assumed to be a conformational activation mechanism (58, 59). Similarly, both heparin and vitronectin enhance the inhibition rate constant of thrombin by PAI-1 up to 200-fold (60, 61). The vitronectin-induced acceleration of thrombin inhibition by PAI-1 has been suggested not to be due to a
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Some serpins require secondary interactions for efficient inhibition of the target proteinase. Exosites on heparin cofactor II (residues 54-75) (64) and on protease nexin 1 (65) interact with the anion binding site of thrombin. The interaction between the amino-terminal side of plasmin and the carboxy-terminal side of \( \alpha_2 \)-antiplasmin has been shown to contribute significantly to the rapid inhibition of plasmin (66). Additional interaction sites between PAI-1 and t-PA that stabilize the reversible complex have been suggested (67-70). The residues P4', P5' and P9' of PAI-1 were assumed to be important for this interaction (70). However, replacement of residue P9'(Asp) with an Arg resulted in a mutant that was completely inactive towards t-PA and u-PA (71). On the other hand, substitution of the P4'(Glu) and P5'(Glu) with an Arg resulted only in a slight decrease of the inhibition rate constants (i.e. \( k_2 = 9.2 \times 10^5 \text{M}^{-1}\text{s}^{-1} \) and \( 7.2 \times 10^5 \text{M}^{-1}\text{s}^{-1} \), respectively vs \( k_2 = 1.4 \times 10^6 \text{M}^{-1}\text{s}^{-1} \) for wtPAI-1 towards t-PA (71, 72).

A recent study, using \( \alpha_1 \)-antichymotrypsin as a scaffold to generate chimeras containing the reactive site loop (P10-P13') of either antithrombin III, \( \alpha_1 \)-antitrypsin or protease nexin 1, revealed that, apart from the PAI-1 residues and the secondary binding sites, also intramolecular interactions control the target specificity of a serpin (73).

**Inhibitor versus Substrate**

**Mechanistic Considerations**

The serpins can be divided in two groups: the active inhibitory serpins (e.g. \( \alpha_1 \)-antitrypsin, \( \alpha_1 \)-antichymotrypsin, PAI-1, antithrombin III, ...) and the non-inhibitory serpins (e.g. ovalbumin, angiotensinogen, maspin, ... (10, 74) (cfr. Table 1). Inhibitory serpins interact through formation of a 1:1 stoichiometric reversible complex with their target proteinases followed by covalent binding between the hydroxyl group of the active-site serine residue of the proteinase and the carbonyl group of the P1 residue at the reactive site (bait region) of the serpin. Typically, this bait residue (P1) mimics the normal substrate of the target proteinase (75). Non-inhibitory serpins react with their target proteinases resulting in a cleavage of the PAI1’ bond but, in contrast to the inhibitory serpins, without formation of a covalent complex and without inhibition of the proteinase. In general, the inhibitory serpins appear to have a more flexible reactive site loop due to the presence of alamines in the P13 to P9 region. The non-inhibitory serpins harbor rather bulky or charged residues in this hinge region (23) (Table 2). It has been demonstrated subsequently that the decreased kinetics of insertion (through bulky or charged residues in the proximal hinge region (P15-P10) of the reactive site loop) are associated with an increased substrate behaviour (76-78).

Creating a disulfide bridge between the Pro(361,P3')→Cys and residue Ser(283,s2C)→Cys in \( \alpha_1 \)-antitrypsin, revealed that for inhibitory activity there is no requirement for mobility in the distal hinge region (P7’-P12’) of the reactive site loop of the serpins (79).

Thus, inhibitory serpins form SDS-stable proteinase/serpin complexes with their target proteinases due to the formation of an ester bond (158) between the carbonyl group of the P1 residue of the serpin and the hydroxyl group of the active-site serine residue of the target proteinase thereby inhibiting the activity of the target proteinase. In this covalent complex, the inhibitor is cleaved at the PAI1’ site (80-83) revealing that the function of a serpin is restricted to a single encounter with its target proteinase. Analysis of the reaction products formed upon interaction between serpins and their target proteinases has revealed that often, in addition to the formation of a covalent complex, also a cleaved derivative of the serpin is formed.

It is generally accepted (84-87) that, irrespective of the final products (EF for the inhibitory interaction or Fi for the substrate interaction; Fig. 4), initially a noncovalent reversible Michaelis complex (EI) is formed between the inhibitor (I) and its target proteinase (E), followed by the formation of an acyl intermediate (EI'). Two possible hypotheses have been proposed to explain the “inhibitor” vs “substrate”-type of interactions between serpins and their target proteinase (Fig. 4a and b): (1) According to the branched pathway hypothesis (Fig. 4a), the acyl intermediate (EI') forms a branching point during the interaction process between a serpin and its target proteinase. This intermediate complex can result either in the formation of a covalent complex (irreversible, E; inactive) or in the regeneration of the proteinase (E)
and the irreversible cleavage of the serpin (F) (Fig. 4a). According to this hypothesis, changes in external conditions (e.g. pH, salt, temperature or cofactors) may influence this branching point resulting in a shift of the ratio between $k_1$ and $k_2$, ultimately resulting in a shift of the ratio between complexed (EF) and cleaved reaction products (F) (47, 85 to 87).

(2) According to initial conformation pathway two distinct initial conformations ["substrate" (I$_{s}$) vs "active" (I$_{a}$)] of the serpin exist predestined to be cleaved without (F) or with formation of a stable complex (EF), respectively (Fig. 4b). Indeed, for PAI-1 a distinct intact reactive conformation was isolated that, in contrast to the inhibitory conformation, does not form stable complexes with its target proteinase but is cleaved at the P1P1' bond (= 'substrate'-type of interaction) (27, 55, 88-91) without inhibition of the target proteinase. In addition, the spontaneous conversion of the active inhibitory form into a distinct substrate form for other PAI-1 variants (PAI-1-Ser(337,P10)→Pro and PAI-1-Asn(329,P18)→Pro) provided further evidence for this alternative reaction scheme (55). Similar conversions were suggested in PAI-1 variants in which mutations at position 125 (Asp→Lys) or 133 (Arg→Asp) were introduced (159). According to the mechanism proposed in Fig. 4b, changes in external conditions (e.g. pH, salt concentration, temperature or cofactors) may induce subtle conformational changes in the serpin resulting in shifts in the ratio between the active (I$_{a}$) and the substrate conformation (I$_{s}$) which in turn results in a shift of the ratio between complexed and cleaved reaction products (55, 91).

The observation that Triton X-100 and other amphiphilic compounds induce time-dependent conformational changes in PAI-1 (i.e. conversion of the active form into a non-reactive form via a substrate-like intermediate) prior to interaction with its target proteinase substantiates the hypothesis of the existence of distinct initial conformations of serpins determining the outcome of the reaction products (92).

Taken together, combination of all currently available data results in an overall reaction scheme as indicated in Fig. 5 taking into account both hypotheses to explain the variations in ratios between cleaved and complexed reaction products.

Neutralization of Serpin Activity through Induction of Substrate Behaviour

Incubation of serpins (e.g. α$_1$-antitrypsin, antithrombin III, PAI-1) with a peptide corresponding to their respective P14-P1 (or P14-P7, or P14-P10) results in an inactivation of the serpin. This lack of activity is either due to an increased substrate behaviour (93-95, 160) or a transition to a non-reactive form (96).

Monoclonal antibodies directed against a neoantigenic epitope (comprising residues P12-P8) in the human antithrombin/thrombin complex have been shown to convert the inhibitory pathway into a substrate pathway (97, 98). This was suggested to be due to a deacylation of an intermediate enzyme-inhibitor complex. Alternatively, monoclonal antibodies directed against the α$\beta$-helix F in PAI-1 have been shown to induce substrate properties through conformational changes resulting in a stabilisation of the α$\beta$-helix F (99).

Mutant Variants of Serpins with an Increased Substrate Behaviour

The reactive site loop is hinged to the protein core at each end. The P15 to P10 residues, situated on the N-terminal part of the reactive site loop, form the proximal hinge whereas the strand s1C together with the turn connecting s1C with s4B form the distal hinge (P7$^-$ to P12$^+$) of the reactive site loop. Several naturally occurring mutants of inhibitory serpins have been described (for review see references 100 and 101) where either point mutations [C1-inhibitor We (102), C1-inhibitor Ma (103), antithrombin III Hamilton (104) and antithrombin III Cambridge-I (105)] or alanine insertions [α$_1$-antiplasmin Enschede (106)] in the proximal hinge region resulted in inactive serpins with substrate properties. Mutagenesis studies suggested that either the charge of residue P14 (78, 107) or "structural" restrictions in the P5 to P18 region (27, 55, 108) alter the functional behaviour of PAI-1 from an inhibitor into a substrate. Alternatively, shortening the reactive site loop by deletion of the P6-P4 or P9-P4 region in PAI-1 also results in inactive variants with an increased substrate behaviour (109). Taken together, all data on serpin variants with increased or exclusive substrate properties support the hypothesis that the kinetics of insertion may determine the functional behaviour of a serpin (76-78).

Polymerization of Serpins

PAI-2 is the only serpin known to spontaneously form polymers during incubation at room temperature (110).

Mutations in the distal hinge region (P7$^-$-P12$^+$) of the reactive site loop do not alter the functional behaviour of a serpin but often result in variants with impaired inhibitory activity due to polymerization. In C1-inhibitor, point mutations in the distal hinge region at position P8$'$ (Val→Met) (111) or position P10$'$ (Phe→Ser) (111, 112) as well as in the proximal hinge region at position P10 (Ala→Thr) (113) result in variants with decreased inhibitory properties due to polymerization. The Z-variant of α$_1$-antitrypsin [Glu(342, P17)→Lys] (114) as well as the antithrombin III Rouen-VI variant [Asn(187)→Asp] (115) harbor a mutation that interferes with the stability of sheet A, also leading to polymerization. Eventually, polymerization results in a decreased concentration of active circulating serpin (112, 113, 115-119) and is the underlying cause of several diseases, including liver cirrhosis, angiodema, emphyesema and thromboembolism. A similar polymerization can be induced in native inhibitory serpins by limited thermal denaturation or mild treatment with denaturants (110, 119, 120). Two mechanisms of loop-sheet polymerization have been proposed. The loop-sheet A polymerization mechanism (93, 118, 121) suggests the insertion of part of the reactive site loop of one molecule into the β-sheet A from another molecule ("head-to-tail"). However, the crystal structure of an antithrombin III dimer (31, 122), consisting of an active and a latent molecule, suggested that the reactive site loop of the active molecule inserts into β-sheet C of the latent molecule suggesting another polymerization mechanism, i.e. loop-sheet C polymerization ("head-to-head") (31, 101, 119). Indeed, in the latent conformation of a serpin, insertion of the reactive site loop into β-sheet A results in a "detachment" of the strand s1C from β-sheet C which subsequently can be replaced by the corresponding region of the reactive site loop of another molecule.

Fluorescence measurements on α$_1$-antitrypsin revealed that the mobility of strand s1C is essential to polymerization (123). Chang and coworkers (124) supported these findings by engineering an active α$_1$-antitrypsin mutant [Pro(361,s1C)→Cys and Ser(283,s2C)→Cys] in which a disulfide bond between strand s1C and s2C was made. Their study indicated that the mobility of strand s1C is a common feature of both loop A-sheet and loop C-sheet polymerization.

Increasing the Functional Stability

PAI-1 is the only serpin that converts into a more stable latent conformation under physiological conditions. However, mild denaturating...
conditions can induce a form with a similar structure and similar properties in other serpins (23, 119, 125). Several conditions, such as low temperature, low pH and high salt concentrations can reduce the rate of latency transition in PAI-1 (126). Kvasmann et al. (95) have linked the acid stabilization of the active form of PAI-1 to protonation of the His143 residue located on α-helix F. Several attempts have been made to increase the functional stability of PAI-1 by site-directed mutagenesis or by random mutagenesis. Latent PAI-1 harbors a salt bridge between Arg(30,s6B) and Glu(350,P4) of the reactive site loop. Lawrence and coworkers (127) prevented formation of this salt bridge by introducing mutations at either one or both of these positions. The stability of these mutants was at maximum two-fold increased indicating that although this salt bridge may play a role in determining the functional half-life of PAI-1, also other factors contribute significantly to the lability of PAI-1. At random mutagenesis (128) combined with a phage-displayed based selection system allowed the isolation of a PAI-1 variant with a 72-fold increase in functional stability. This PAI-1 mutant was found to contain four mutations [Asn(150,thFs3A), Lys(154,thFs3A), Thr(333, P8) and Val(197,ts4Cs3C)]. Substitution of either one of the P-even residues in the active site loop with glutamate or construction of a disulfide bridge between Val(197,ts4Cs3C)→Cys and an appended Cys-Val-Lys C-terminus, all yielded mutants with an increased half-life [up to 8-fold for the Thr(333, P8)→Glu mutant] except for the Glu substitution at position P14 (107). A recent study identified a positively charged region in PAI-1 (i.e. residues Arg186, Arg187, His190, Lys191) that contributes significantly to its functional stability (129). In addition, a residue localized in s2B (i.e. Tyr221) was found to play a role in the stability of PAI-1 (161).

Interaction with Ligands and Localization of Ligand Binding Sites

A number of ligands specifically interacting with serpins have been identified. In general, these interactions serve either to localize the serpin at specific target sites and/or to modulate their activity.

Negatively charged glycosaminoglycans (i.e. heparin, heparin sulfate, dermatan sulfate, chondroitin sulfates, ... ) increase the rate of inhibition at which the heparin-activatable serpins (i.e. antithrombin III, heparin cofactor II, protein C-inhibitor, protease nexin I) inactivate their target proteinases. Charged basic residues as Arg and Lys have been identified as heparin binding sites. Except for protein C inhibitor, these heparin binding sites are localized on α-helix D (10, 130). The residues Lys11, Arg13, Arg24, Arg47, Lys125, Arg129 and Arg 145 (either located in hD or in its close proximity) have been identified as the heparin binding site of antithrombin III (131). Based on these recent data, it is proposed that heparin breaks the intramolecular hD-sheet B salt bridges, facilitates the s123AhDEF movement and generates a conformation that can easily form an inhibitory complex. In protein C inhibitor, α-helix H and the amino acids preceding α-helix A form the positively charged region for heparin binding (132, 133).

For PAI-1 a specific interaction with vitronectin has been observed (134, 135). This binding results in a two-fold stabilization of PAI-1 (134), in a localization of active PAI-1 in the extracellular matrix (136) and may also contribute to its target specificity (60, 61). Studies with monoclonal antibodies (137) suggested that the vitronectin binding site of PAI-1 is located between residues 128 and 145 (hF). However, this could not be confirmed in another study (99) using different monoclonal antibodies binding to a similar region in PAI-1 (i.e. residues 128-156, hF-thFs3A). On the other hand, random mutagenesis (138), PAI-1/PAI-2 chimeras and peptide mapping (139) revealed that the clustered group of residues 109, 110, 116 and 123 as well as residue 55 are implicated in binding of active PAI-1 to vitronectin. These residues are located in helices C and E, which are in close proximity with the β-sheet A. The latter may well explain the stabilizing properties of vitronectin bound to PAI-1. Binding of PAI-1 to vitronectin has also been suggested to induce a conformational change in the vitronectin moiety resulting in an enhanced thrombin binding to vitronectin and an increased rate of inhibition by PAI-1 (140). PAI-1 has also been shown to bind to fibrin possibly through residues 284-294 (hI) and residues 110-145 (hE, s1A, hF) (61). The hormone binding regions of thyroxine binding globulin and corticosteroid binding globulin are on the interior surfaces of β-sheets B and C (141, 142). The DNA binding region of α,-antichymotrypsin consisting of 5 lysines is located at the turn of strand s3B and s4B (17, 143). The physiological role of these DNA binding properties is not known.

Lysine residues located at the carboxyterminal end of α,-antiplasmin interact with the lysine binding region of plasmin (66, 144, 145). Since this region of plasmin is responsible for its interaction with fibrin, fibrin-bound plasmin is resistant to inhibition by α,-antiplasmin. A second ligand binding site on α,-antiplasmin is the second residue (Glu) at the aminoterminal end which interacts with the α,A-chain of fibrin. This results in a covalent immobilization of α,-antiplasmin and subsequently a stabilization of the blood clot.

Complexed serpins are more rapidly cleared than the native serpins (146). The serpin-enzyme complex receptor (SEC-receptor) (147) as well as the receptors of the low-density lipoprotein receptor (LDLR) family, especially α,-macroglobulin receptor/low-density-lipoprotein-receptor-related protein (α-MR/LRP) and very-low-density-related-receptor protein (VLDLR) (cf. reviews 146, 148) have been shown to mediate endocytosis of serine proteinase/serpin complexes. Characterization of the binding specificities of α-MR/LRP and VLDLR revealed that both the serine proteinase and the serpin moiety contribute to the specificity (149). The positively charged heparin binding area of the serine proteinase/serpin complexes was suggested to be the binding site to the receptor (146). However, the thrombin/heparin cofactor II complex containing two heparin binding sites was unable to bind to the receptor (150). Rodenburg et al. (1998) demonstrated that four basic residues (Arg78, Lys82, Arg120, Lys124) located in α-helix D (= the heparin binding site) and β-strand 1A are involved in the binding of the u-PA/PAI-1 complex to both the α-MR/LRP and VLDLR receptor (149, 151).

Serpín/Proteinase Complex

Over the last decade numerous studies have provided more insights into the mechanism of interaction between serpins and their target proteinases. However, one key feature, i.e. the exact structure of the ultimately formed serpin/proteinase complex cannot be addressed yet because of the lack of X-ray data on such a structure. In the absence of this information, different models for the serpin/proteinase complex have been proposed based on the available structures of the individual constituents, combined with biochemical observations and/or cross-linking experiments.
Convincing evidence has been obtained that in the complex, the P1P1’ bond of the serpin is cleaved and that a stable acyl bond is formed between P1 and the active-site serine residue of the proteinase (80-83, 158). The necessity of full loop insertion upon complex formation was proposed based on the crystal structures of the cleaved inhibitory serpins (152). Full loop insertion would require the movement of the proteinase to the opposite site of the serpin. However, recent studies suggested that only a partial loop insertion occurs in the proteinase/inhibitor complex. In one study, the initial PAI-1/proteinase complex as well as the stable covalent complex has been modelled (153). In this model, the reactive site loop of PAI-1 is completely exposed before complex formation to facilitate the interaction with the catalytic site of the proteinase. The subsequent formation of a stable complex is proposed to require cleavage followed by insertion of the N-terminal part of the reactive site loop up to P7. Stratikos and Gettins (154) used fluorescence resonance energy transfer to study the differences between a noncovalent Michaelis-like complex and a stable complex. Their study revealed that the proteinase has to make a large move relative to the inhibitor upon formation of the noncovalent Michaelis-like complex to the kinetically stable acyl-enzyme complex. Wilczynska et al. (155) provided evidence that the proteinase prevents full insertion of the reactive site loop. The ultimate position of the proteinase in the stable complex is stabilized by multiple interactions with the serpin. This is in agreement with the findings of Plotnick and coworkers (156) who observed an apparent distortion of the catalytic site of chymotrypsin in the serpin/proteinase complex by using proton nuclear magnetic resonance spectroscopy. Based upon all these data, the following general reaction mechanism between serpins and their target proteinase is proposed (157). The reactive site of a serpin is cleaved and the P1 residue forms a covalent bond with the catalytic site of the proteinase. The P-residues of the reactive site loop (covalently linked to the proteinase through the P1 residue) partly insert into the β-sheet A. Due to this insertion, the proteinase is moved from the docking position to a position in the middle of β-sheet A of the serpin thereby distorting the catalytic site of the proteinase and yielding a stable acyl-bonded serpin/proteinase complex.

**Conclusions**

Although the crystal structure of several serpins has been elucidated, the necessity of pre-insertion, prior to interaction with the target proteinase, is still a matter of debate. It is clear that, in contrast to previous hypotheses, a lack of inhibitory activity is not associated with a lack of insertion of the reactive site loop. In the current concept the kinetics of insertion play an important role in the functional behaviour of a serpin.

The molecular explanation for the occurrence of substrate behaviour of serpins is still a controversy. According to the branched pathway, the ratio between the reaction products (complex versus cleaved) partly insert into the β-sheet A of the serpin thereby distorting the catalytic site of the proteinase and yielding a stable acyl-bonded serpin/proteinase complex.

Even though the target specificity of the serpins is mainly determined by the P1 residue, residues remote of the P1P1’ region can also alter this specificity. In addition, secondary binding sites, cofactors and intramolecular interactions have been shown to contribute to the target specificity of a serpin.

Recently, different states of complexes between serpins and their target proteinase have been modelled. The current concept is based upon a cleavage of the P1P1’ bond, a partial insertion of the P-residues of the reactive site loop and a concomitant movement of the target proteinase to the middle of the inhibitor. The ultimate answer on the structural features of a serpin/proteinase complex will only be provided by elucidation of the 3-dimensional structure of such a complex.

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