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Structural Basis for Serpin Inhibitor Activity

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ABSTRACT **The mechanism of formation and the structures of serpin–inhibitor complexes are not completely understood, despite detailed knowledge of the structures of a number of cleaved and uncleaved inhibitor, noninhibitor, and latent serpins. It has been proposed from comparison of inhibitor and noninhibitor serpins in the cleaved and uncleaved forms that insertion of strand s4A into preexisting β-sheet A is a requirement for serpin inhibitor activity. We have investigated the role of this strand in formation of serpin–proteinase complexes and in serpin inhibitor activity through homology modeling of wild type inhibitor, mutant substrate, and latent serpins, and of putative serpin–proteinase complexes. These models explain the high stability of the complexes and provide an understanding of substrate behavior in serpins with point mutations in s4A and of latency in plasmingoen activator inhibitor I.** © 1995 Wiley-Liss, Inc.

Key words: serpin–proteinase complex, mutants, deamidation, α-helix–β-sheet conversion, homology modeling

INTRODUCTION

The serpin family of proteins consists primarily of specific proteinase inhibitors, which slowly form tight binding complexes with their target proteinases.[1–3] A smaller number of noninhibitor serpins, as well as point mutants of inhibitor serpins, are attacked by proteinases without forming stable enzyme–inhibitor complexes, but instead are cleaved like substrates of the proteinase. Previous studies of the structure of the cleaved noninhibitor serpin, plakalbumin,[4] the proteolyzed form of ovalbumin, associated the lack of inhibitor activity in ovalbumin with a failure of the cleaved strand (s4A) to insert into preexisting β-sheet A,* as observed in the cleaved forms of α_1-proteinase inhibitor,[5] antithrombin III,[7] antichymotrypsin,[8] and leukocyte elastase inhibitor.[9] This failure of the cleaved strand in plakalbumin to insert into β-sheet A was attributed to the identity of residue 345 at the P14† position, which is arginine in ovalbumin and glutamic acid in the noninhibitor angiotensinogen, in contrast to small, uncharged residues in the inhibitor serpins. The size and charge of arginine appear to prevent it from inserting into the hydrophobic interior of the ovalbumin molecule in the cleaved, strand-inserted conformation.

The role of the different conformations of the reactive site target segment, which becomes s4A in cleaved serpins, is not yet completely understood. The structures of the noninhibitor ovalbumin and the inhibitor antichymotrypsin show this segment to be an α-helix and a distorted α-helix, respectively, extending from the body of the molecule on two polypeptide stalks.[11,25] The possible association of serpin inhibitor function with β-sheet strand insertion, and thereby with the identity of residue 345 at P14 and also with other even numbered residues amino terminal to the P1 cleavage site, has led to proposals that this strand must be partially inserted into β-sheet sA for the intact serpin to be in its proteinase-susceptible conformation.[12,13] The crystal structures of the uncleaved latent (inactive) form of plasminogen activator inhibitor 1 (PAI1),[15] and of one of two molecules in the asymmetric unit of antithrombin,[16,17] show s4A extensively inserted into β-sheet A, which renders the target residues inaccessible to attack by proteinase. The polymorphism of this strand has been attributed to an intrinsic flexibility

*Sequence numbering and nomenclature of the secondary structural elements are that of Loebermann et al.[5] and Huber and Carrell.[6]

†Numbering of reactive site residues follows that of Schechter and Berger[10] for proteinase substrates in which the P1 residue contributes the carbonyl group of the cleavage site, and residues in the amino terminal direction are denoted P2, P3, etc. and in the carboxyl terminal direction P1′, P2′, P3′, etc.

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inferred from multiple conformations for it in the crystal structure of ovalbumin[12] and from the two conformations observed in intact antithrombin,[16] but others have found no evidence for such flexibility in ovalbumin in solution.[18]

To address some of these questions of serpin function, we have focused on three observations which are general to most members of the serpin family and which we hypothesize are related: (1) the sensitivity of serpin function to point mutations in the s4A chain; (2) the stabilization to denaturation of cleaved relative to uncleaved serpins; (3) the exceptional stability of serpin–proteinase complexes. We have used structure-based models of wild type and point mutant serpins and of hypothetical proteinase–serpin complexes to examine the role of s4A in serpin function. We discuss here possible structures for intermediates implicated in the proposed mechanism for serpin function, and offer an explanation for the differential substrate and inhibitor activities of some serpins and their mutants and a model structure for the highly stable serpin–proteinase complexes.

EXPERIMENTAL

Model Building of Unknown Serpin Structures

Models of antiplasmin, antiplasmin Enschede, C1 inhibitor, C1 inhibitor Ma and Ca/Mo, and plasminogen activator inhibitor I were built on an Evans and Sutherland PS390 with Frodo using as template coordinates for the known structures of cleaved α_1-proteinase inhibitor and ovalbumin in the Brookhaven Protein Database. The amino acid sequence of the serpin to be modelled was substituted for that of α_1-proteinase inhibitor, side chain conformations set, where not in conflict, according to the distributions of Ponder and Richards,[19] and manual adjustments made to minimize bad contacts. In the initial models of the point mutants, conformations of side chains near to the mutant amino acid were manually adjusted and, where possible, hydrogen bonds were formed. Models of antithrombin Hamilton and Cambridge were derived from the crystal structure coordinates of antithrombin kindly provided by Dino Moras.

Models of the complexes of elastase–α_1-proteinase inhibitor and plasmin–antiplasmin were based on crystallographic and/or model-based coordinates obtained as described above, using trypsin as a template for the plasmin model. Both the plasmin and the antiplasmin model structures are truncated to only those segments of their sequences which can be mapped to the template structures, and are thus shorter than their biologically active forms. The energy minimized structures for proteinase and strand-inserted serpin were docked using INSIGHT (Biosym Technologies, San Diego, CA) subject to the constraint imposed by the covalent geometry of the acyl linkage between the catalytic serine O_γ and the terminal carbonyl carbon of the terminus of s4A. Initial models were refined using the BORN module and the united atom force field of AMBER 3.0 (licensed from the University of California, San Francisco) in 100 cycles of steepest descent minimization followed by conjugate gradient minimization until the rms of the norm of the gradient was less than 0.09. The distance-dependent dielectric of 1.0 was used for all minimizations. Minimizations were on either an IBM powerstation model 730 or a DEC Alpha AXP workstation.

To examine the contact interface between plasmin and both antiplasmin and antiplasmin Enschede, we used the HINT program (EDUSOFT, Ashland, VA) interfaced to INSIGHT II. Missing protons were added to the Amber minimized structures of the complexes, and the resulting structures were minimized with the DISCOVER module of INSIGHT II. These minimized structures were then analyzed with HINT to compute hydrophobic and polar interaction surfaces between the serpin and protease.

Circular Dichroism Measurements

The s4A model peptide of antiplasmin (acetylEVGVEAAAATSIAZSR) and antiplasmin Enschede(acetylEVGVEAAAAATSIAZSR)(Z = norleucine) was synthesized and its solution conformation determined by circular dichroism measurements. Solutions of 0.42 mM peptide in phosphate-buffered saline at pH 6.8 were measured at temperatures of 4, 20, and 37°C using a Jasco spectrometer.

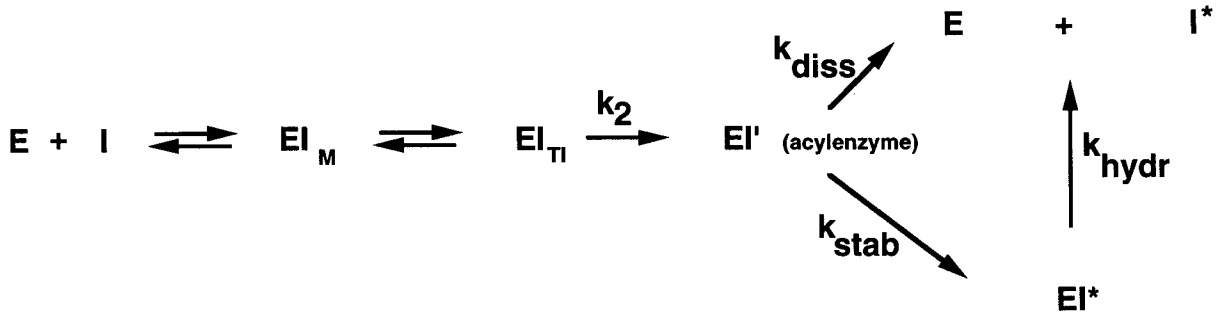
RESULTS AND DISCUSSION

The Serpin Inhibition Mechanism

Current understanding of the mechanism of serpin inhibition and of the structures of pertinent forms of the serpins and their complexes with proteinases is still incomplete. Olson,[20] Rubin et al.,[21] Patston et al.,[22] Cooperman et al.,[23] and Schechter et al.[24] have discussed a suicide substrate mechanism for serpin inhibition of target proteinases, which is consistent with available kinetic data for these and other serpin inhibition reactions (Scheme 1).

In this mechanism, serpin products partition between cleaved (I*, i.e., substrate) serpins and stable enzyme–serpin complexes (EI*) in a ratio represented by the stoichiometry of inhibition ($SI = 1 + k_{diss}/k_{stab}$). The rate constants k_2, k_{diss}, and k_{stab} may all influence the relative amounts of cleaved serpin (I*) and stable proteinase–serpin complex (EI*), and are likely to vary among the different serpin–proteinase pairs. Initially, the level of proteinase activity will be determined by the value of SI, but on a longer and, in some cases, more physiologically relevant time scale, it will be determined by the value of k_{hydr}.

The nature of the initial binding of proteinase to serpin to form the EI complex is unclear. Three structures of intact serpins are known (ovalbumin,[11]



Scheme 1. Proposed suicide inhibitor mechanism for serpins. EI_M is a Michaelis complex, EI_{TI} is a tetrahedral intermediate, EI′ is the acyl enzyme, and EI* is the stable complex of proteinase with fully inserted s4A.

α_1-antichymotrypsin,[25] and antithrombin[16,17]). In the first two of these intact serpins, the structure of the reactive site s4A segment is helical or distorted helix, and differs from that of other small protein inhibitors of serine proteinases and from the known β-sheet conformation of peptide substrates bound to serine proteinases. In the intact antithrombin structure, the two unique molecules differ in the conformation of the reactive site segment. One is fully inserted into s4A and the other appears to be partially inserted, though the electron density for it is uncertain. This polymorphism in the reactive site segment may reflect a mixed population of α-helical and partially inserted β-sheet conformations, only the latter of which binds proteinase. Alternatively, the binding of proteinase to this metastable segment in the α-helical conformation may induce a β-sheet conformation around P1, like that observed in complexes of extended peptide substrates with serine proteinases. The structures of intact, latent, and cleaved serpins support proposals that this strand can adopt either an α-helical or β-sheet conformation with varying extents of insertion into β-sheet A. There is evidence that proteinase-susceptible sites undergo significant conformational change, and must therefore have a degree of flexibility which permits them to attain the optimal conformation for cleavage by the proteinase.[26–29] In this respect, the serpins resemble other susceptible sites of limited proteolysis, but differ from the preformed, optimal configuration which occurs in other protein proteinase inhibitors.

There is evidence supporting the existence of an initial noncovalent EI complex between trypsin or chymotrypsin and antiplasmin,[30] which is dissociable to intact serpin and active proteinase[31] in the presence of α_2-macroglobulin. There is also evidence for a tetrahedral intermediate linked complex of α_1-proteinase inhibitor with porcine pancreatic elastase, which has been suggested to be in equilibrium with the EI Michaelis complex.[32] A tetrahedral intermediate complex may be stable, but we think it is more likely that the more stable acyl-enzyme forms at the partition step of the mechanism (EI′). Stable complexes of proteinases with serpins have been isolated and identified,[21,32–43] most frequently by SDS-PAGE electrophoresis, implying that they have unusually high stability indicative of covalent linkage.

The plakalbumin structure and the lack of inhibitor activity in ovalbumin are consistent with a requirement for insertion of s4A for inhibitor activity. The growing number of dysfunctional mutant serpins with sequence changes in s4A, chemical modification studies,[47] exogenous peptide strand-insertion complexes,[48,49] limited proteolysis,[17,50] and limited denaturation[12] all implicate the insertion of strand s4A in the inhibitor activity of the serpins. It has been proposed[12,13] that these data reflect a requirement for partial insertion of s4A, from residues P14 through P10, in order for the serpin to assume its proteinase-susceptible binding site. However, it has been pointed out that for a P10 (Gly→Pro) hinge region mutant of α_1-proteinase inhibitor of s4A, which transforms the serpin into a substrate, it is the stability of the proteinase–serpin complex and not its association rate which is affected.[14]

Comparison of the stabilities of intact and cleaved, fully strand inserted inhibitor serpins has been done by both calorimetry and by spectroscopic monitoring of transitions in varying concentrations of denaturant.[3,45,46] Carrell et al.[12] and Zhong (cited in Rubin et al.[51]) have found evidence for forms of intermediate stability between native and cleaved, which they ascribe to partial insertion of s4A, though the extent of insertion could not be determined. The denaturation studies of Lennick et al.[42] on C1 inhibitor in complex with C1s, support an extremely stable complex resistant to both 6 M guanidine hydrochloride and to temperatures up to 90°. Most of the evidence for high stability in the proteinase–serpin complexes is their observation in denaturing SDS gel electrophoresis, where survival of both detergent and high temperature are consistent with extreme stability of the complex. While direct comparisons of complex stability with that of native and cleaved forms are lacking, the detection of proteinase–serpin complex under rigorous denaturation conditions more closely resembles the latter.

We propose that the highly stable serpin–proteinase complexes exist in a fully strand inserted form like that of the cleaved inhibitor serpins, and that this complex forms in the irreversible step from EI′ to EI*. Formation of this complex would occur after formation of acyl enzyme with migration of covalently linked proteinase from one pole of the serpin to the opposite one, which would be mediated by the insertion of strand s4A. The stability of this complex to hydrolysis would be determined by the degree of protection of the acyl linkage from solvent nucleophiles during and after strand migration. Such protection in the terminal complex would be realized simultaneously with further increase in complex stability, if extensive interactions between the serpin and covalently bound proteinase occur at the opposite end of the serpin molecule from that at which initial attack on the reactive site occurs. For those serpin–proteinase pairs with SI near to 1.0, the stability of the EI* complex, and therefore the level of active proteinase, will be controlled by k_{hydr}. This rate constant will be determined by the interactions of the proteinase in its new site bound to the serpin, and on the protection of the acyl enzyme linkage from hydrolytic breakdown. The cases of noninhibitory ovalbumin and of some of the point mutants can be understood as arising from size and charge exclusion effects which hinder or prevent insertion of s4A and so expose the acyl linkage to hydrolysis before insertion and proteinase migration can occur.

The hypothesis proposed here for the structure of stable proteinase–serpin complex does not preclude partial insertion of s4A in intact serpins as a prerequisite for proteinase attack and formation of the initial noncovalent complex. However, there is little direct evidence to support this under conditions of proteinase inhibition, and we think it more likely that insertion of s4A is a consequence rather than a cause of proteinase attack on the serpin reactive site, as suggested by Carrell et al.[17] Bjork et al.[49,52] have proposed a similar serpin–proteinase complex structure to that proposed here based on kinetic and immunologic activities of antithrombin. In these studies, it was proposed that the proteinase moves to a different position from that at which attack initially occurs, but only limited insertion of s4A was hypothesized. We believe that the exceptionally high stability of the serpin–proteinase complexes, which is consistent with models of serpin–proteinase complex described here, is more consistent with full insertion of s4A into β-sheet A.

Models of Serpins and Their Complexes With Target Proteinases

To examine the hypothesis that the stability of postcleavage complexes of serpins with target proteinases depends upon a stable, fully inserted s4A covalently linked to proteinase, we built model structures of some serpins and their mutants, and of complexes of serpins with their target proteinases, based on known serpin and proteinase structures. In all of these structures, s4A is in the fully strand-inserted conformation, and in the complexes the proteinase is bound in acyl linkage to the carboxyl terminus of s4A. In this conformation, we maximized the number of stabilizing contacts between the serpin and the proteinase, while simultaneously minimizing the exposure of the acyl linkage to solvent water. For each of the point mutations in s4A, we sought structural explanations for the effects of the amino acid substitution on the stability of the inserted s4A in β-sheet A. Our canonical model of the α_1-proteinase inhibitor complex with human neutrophil elastase is shown in Figure 1.

Table I lists some serpins with point mutations in s4A. The occurrence of dysfunctional mutations at the P10 and P12 positions of s4A, and of the oversized and charged amino acids at P14 in ovalbumin and angiotensinogen, is consistent with the inferred requirement for insertion of s4A into β-sheet A for inhibitory activity of the serpins. If strand insertion is required for inhibitor activity, amino acids at the P even positions of s4A must be of a size and polarity which can pack into the interior of the serpin structure beneath β-sheet A. It seems unlikely that amino acid changes at positions P10, P12, and P14, some of which are quite conservative, could affect serpin function by altering the conformation around the scissile bond of the serpin at P1. We infer that these sequence changes alter serpin function at a step subsequent to the cleavage at the EI′ intermediate.

While ovalbumin, angiotensinogen, and some of the serpin mutants can be easily explained by destabilization of s4A in its inserted conformation due to the size and charge of the replacement amino acid, some of the other point mutants are not so easily rationalized by this simple explanation. We have examined these mutations for clues as to how they might affect the kinetics of formation and the stability of s4A in β-sheet A. The rate of strand insertion will be modulated by the barrier to opening strands s3A and s5A to accept s4A and by the stability of s4A in this inserted conformation. A lowered barrier can lead to premature insertion of s4A, either partial or complete, the latter leading to complete loss of susceptibility to proteinase due to disruption of the reactive site conformation, as occurs in latent plasminogen activator inhibitor I and antithrombin. Premature partial insertion or a decrease in the rate of s4A insertion, could expose the acyl linkage in the complex between proteinase and serpin to hydrolysis, resulting in the regeneration of proteinase and production of cleaved substrate serpin (i.e., increase in SI). Either of these latter possibilities could occur as a result of sequence changes in the inward facing even numbered residues of s4A as is observed in mutant serpins.

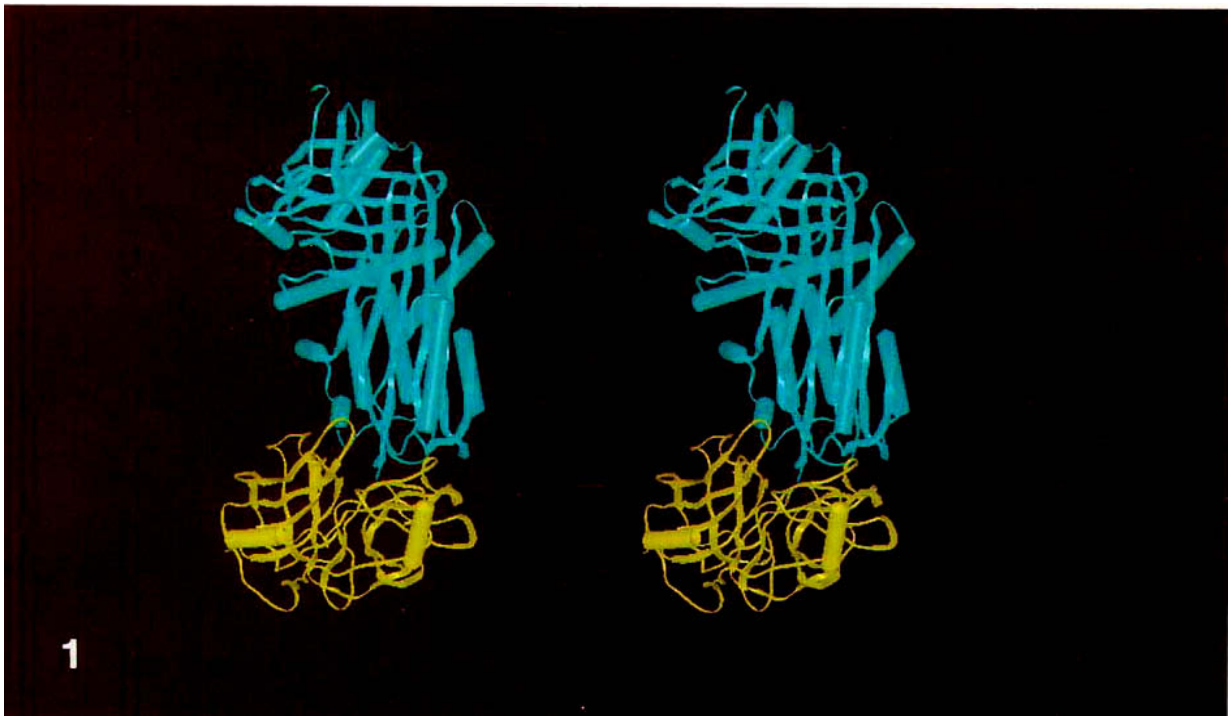


Fig. 1. Stereo views of models of hypothetical complex between human neutrophil elastase and α_1-proteinase inhibitor. Serpin is in blue and proteinase yellow.

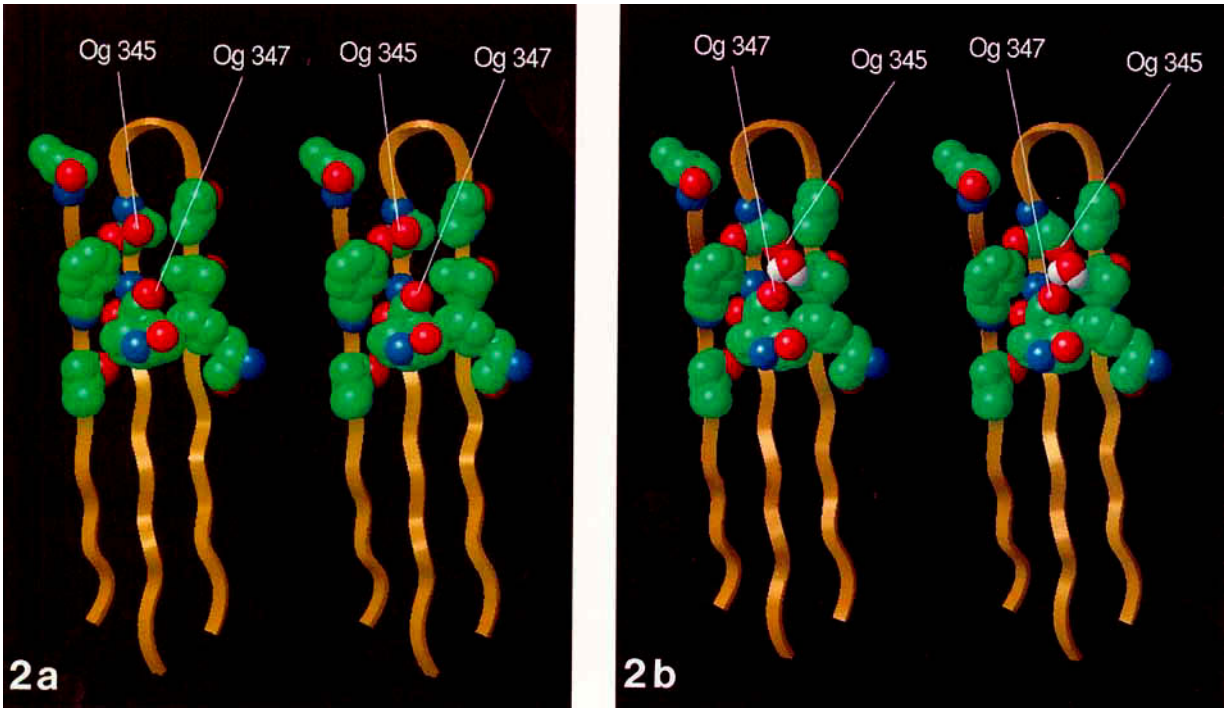


Fig. 2. Stereo figure of model of environment of residue 347 in the strand-inserted conformation of antithrombin Hamilton. View is of underside (interior facing) of β-sheet A for (**a**) Ala→Thr substitution and (**b**) Ala→Thr substitution with water molecule.

TABLE I. Serpin Point Mutations in β-Sheet Strand s4A*

Exterior solvent side

Interior protein side

	P14	12	10	8	6	4	2	1	Activity
Antithrombin									
Hamilton		T							S
Glasgow		↑							
		A							
Cambridge			P						S
Sudbury			↑						
Charleville			A						
Vicenza									
Cambridge II			S						I↓
			↑						
			A						
					V				Venous thrombosis
					↑				
					A				
C1 inhibitor									
MA		E							S
		↑							
		A							
CA		T							S
MO		↑							
		A							
Antiplasmin									
Enschede			A						S
			↑						
α_1-Proteinase inhibitor				E				R	I thrombin
				↑				↑	
				M				M	
	R							R	S thrombin
	↑							↑	
	T							M	
		T							I
		↑							
		A							
			P						S
			↑						
			G						
	R								S↑ I↓
	↑								
	T								

*S, substrate activity; I, inhibitor activity; arrows, increase or decrease relative to wild type.

Antithrombin Mutants

The antithrombin mutants listed in Table I are consistent with a requirement for small nonpolar amino acid side chains at the P10 and P12 residues in s4A. However, model building of mutant antithrombin structures suggests that stereochemical fit alone is not sufficient to confer inhibitor activity.

The antithrombin mutants Cambridge,[53] Charleville,[54] Sudbury,[55] and Vicenza[56] all have an Ala→Pro mutation at P10, and function as substrates of thrombin without formation of stable thrombin–antithrombin complexes. A model of the P10(Pro) mutant shows that the proline side chain would make two close contacts in the strand-inserted form: with Ser-53 and with Phe-384. Both of these could be relieved by minor movements of these residues, and it is more likely that the loss of the hydrogen-bonding main chain –NH– at the mutant Pro in the β-sheet destabilizes s4A in its inserted conformation. This destabilization of the strand-inserted conformation would lead to s4A in its acyl-linked form in complex with thrombin having a longer residence time out of β-sheet A, thus exposing it to solvent hydrolysis of the acyl linkage.

While similar arguments might seem applicable to the substrate antithrombin mutant Hamilton/

Glasgow,[27,58] which have an Ala→Thr mutation at P12, a model of antithrombin Hamilton shows that the Thr side chain of the mutant can be accommodated in the inserted conformation of s4A (Fig. 2). Furthermore, although the O_γ of the Thr side chain in its buried position is too far from the side chain of nearby Ser-345 to make a hydrogen bond, there is sufficient room for a water molecule to bind in this pocket and mediate hydrogen-bond formation between these two hydroxyl groups and thus stabilize the inserted conformation. The enhanced stability suggested by this model of antithrombin Hamilton in the inserted conformation of s4A has been born out by equilibrium physical measurements of antithrombin Hamilton denaturation,[59] which show that antithrombin Hamilton, when cleaved, has a conformation which is at least as stable to denaturation as the cleaved wild type.

Antithrombin Hamilton may fail to function as an inhibitor, either because of premature partial preinsertion of s4A or because of a diminished rate of strand insertion and proteinase migration. There is no clear indication of higher stability to denaturation in uncleaved antithrombin Hamilton,[59] so that if preinsertion of s4A does occur, it must be limited. The absence of simple size and polarity effects of P12 on the decrease in inhibitory activity of antithrombin site mutants[60] is not consistent with equilibrium thermodynamic stability of s4A insertion as the sole determinant of complex stability. P12 replacements which are larger (Ile, Leu, Val), smaller (Gly), and more polar (Gln) than the wild-type Ala all form a larger fraction of stable thrombin–antithrombin complex (though less than the wild-type Ala) than does the Thr mutant. If the uptake of an accompanying water molecule is necessary for strand insertion of s4A, it could decrease the strand insertion rate with increasing order of the reaction. If the rate of this insertion step is slowed, it increases the probability of hydrolysis of the acyl linkage between serpin and proteinase, which would yield cleaved antithrombin product and active thrombin.

C1 Inhibitor Mutants

The C1I Ma mutant (Ala→Glu at P12),[41] like ovalbumin and angiotensinogen, is unable to adopt the s4A strand inserted conformation because of the size and charge of the mutant P12 side chain. This would lead to decreased values of k_2 or k_{stab} or to hydrolysis of EI* (increased k_{hydr} due to a decrease in the rate of strand insertion and exposure of the acyl linkage to solvent).

Models of the C1I Ca/Mo mutants, where P10 is changed from Ala→Thr[61] resemble the antithrombin Hamilton/Glasgow mutants in that Thr can be accommodated in the strand-inserted conformation, and appears likely to be more stable than the wild type. The mutant Thr residue in its buried conformation can hydrogen bond to Ser-53 and/or His-334. Such predicted stability of the mutant over the wild type may explain the tendency of C1I Ca/Mo, but not wild-type C1I, to form multimers in which s4A is inferred to insert into a β-sheet, and the resistance of C1I Ca,Mo to cleavage by C1s proteinase.[61]

Antiplasmin Enschede

The antiplasmin Enschede mutant is unique among the known serpin mutants in s4A in that it is an insertion and not a substitution mutation.[62–64] It also poses a challenge to proposals for the preinsertion of the hinge region of serpins, since it occurs at the P9–P10 position of s4A. While it might be argued that a mutation at this position could disrupt the insertion of s4A at the adjacent more distal positions P10 through P14, this seems unlikely since the extra Ala occurs in the middle of an AlaAlaAla sequence, and thus results in no sequence alteration near to these residues. Furthermore, the effective displacement of downstream (P9→P3) even and odd numbered residues into odd and even numbered positions, respectively, does not lead to replacement of any of the P-even numbered residues by an oversized or polar one. The model of antiplasmin Enschede in this conformation predicts a structure at least as stable as the wild type.

We considered the possibility that the insertion of an extra Ala residue may bias the conformation of the reactive site region toward a more stable α-helix, which would be less able to adopt the β-sheet conformation necessary for bond cleavage and insertion into s4A. Circular dichroic spectra of synthetic peptides for the s4A sequence in both wild-type antiplasmin and antiplasmin–Enschede were examined to determine their solution structures. In both cases, there was no sign of any α-helix structure. We conclude that the nascent s4A reactive site segment of antiplasmin is not intrinsically α-helical, though it may adopt that conformation in the intact antiplasmin reactive site. The fact that antiplasmin Enschede is cleaved as a substrate also argues against the adoption of protected conformations in the reactive site strand of this mutant.

A model of our hypothesized postcleavage, fully strand-inserted complex of plasmin with antiplasmin Enschede compared with the same model complex of wild-type antiplasmin with plasmin suggests that the extra length of strand s4A may expose the serpin–proteinase acyl linkage to hydrolysis in the complex. Comparison of models of plasmin with antiplasmin and with antiplasmin Enschede shows the mutant complex to have 800 Å more solvent-accessible surface than the wild type. Calculation of polar and hydrophobic interaction surfaces between the molecules of these two complexes using HINT[66,67] shows a greater stabilization for the wild-type complex than for the antiplasmin Enschede complex, particularly through hydrophobic interactions. Sta-

bility studies of cleaved antiplasmin Enschede should reveal whether the cleaved form of this mutant serpin is enhanced in stability relative to uncleaved, and thus undergoes full and stable strand insertion like other native serpins.

Latent Plasminogen Activator Inhibitor 1

Plasminogen activator inhibitor 1,[68,69] and to a lesser extent antithrombin,[16,17] can exist as latent forms, which have no inhibitory activity. Denaturation of latent plasminogen activator inhibitor 1 followed by renaturation restores inhibitor activity,[70] which slowly decreases through lapse into the latent form. The structural basis for the lack of activity in latent plasminogen activator inhibitor 1 was revealed in its crystal structure.[15] The reactive site loop of latent plasminogen activator inhibitor 1 from P4 through P15 is inserted as s4A is in the postcleavage form of other serpins, and in this position is inaccessible to attacking proteinase.

We have addressed the question of why plasminogen activator inhibitor 1 is almost unique in the facility with which it can adopt this latent form spontaneously and reversibly, the recovery of inhibitor activity requiring the unfolding of the molecule followed by its refolding into the active native form. Residues 191 and 192 in plasminogen activator inhibitor 1 are Asn–Gly, a sequence known to undergo spontaneous deamidation in a large number of proteins.[71] This deamidation reaction goes through a cyclization of the Asn side chain onto the amido nitrogen of the following Gly to form a succinimide intermediate. This intermediate can be stable or it can break down into two different products: α-Asp or β-Asp, depending on which of the amide bonds of the succinimide is opened by hydrolytic attack. Formation of the nonnative β-Asp disrupts the conformation of the polypeptide chain and renders the sequence susceptible to methylation by an enzyme, protein isoaspartylmethyltransferase, while the other product is a natural L-Asp residue replacing the original Asn.

We propose that the lower barrier to insertion of s4A in uncleaved plasminogen activator inhibitor 1 results from deamidation of Asn-191, which perturbs the interactions which s3A makes with other parts of the structure, particularly the adjacent, parallel s5A. In a model of intact plasminogen activator inhibitor I based upon the ovalbumin structure, the presence of a succinimide intermediate at residues 191 and 192 would clearly destabilize the s3A–s5A β-sheet interactions (Fig. 3). The succinimide disrupts at least one hydrogen bond between s3A and s5A at the upper end of β-sheet A where s4A insertion initiates, and should thus lower the barrier to its insertion. A similar model in which Asn-191 is deamidated to either an α-Asp or isoAsp results in the juxtaposition of the new carboxylate group on s3A and the carboxylate of Glu-339 on s5A (Fig. 3), the repulsion of which could also lower the barrier of s4A insertion between the chains at this point.

Assay of latent PAI1 for isoAsp with L-isoaspartylmethyltransferase gave no methyl group incorporation (Schirch, Goldsmith, and Wright, unpublished). This could indicate either the absence of isoAsp or inaccessibility of the methyl transferase to that site. Our model-building studies of the putative succinimide intermediate and the possible products to which it can give rise as a result of hydrolytic breakdown show that isoAsp is the less likely product to form, due to obstruction of the path of an attacking solvent nucleophile, and that either α-Asp or succinimide is more likely to be the stable products, neither of which would be detected by the methylation assay.

It should also be noted that Gly-192 is a hinge point in the conformational rearrangement which occurs when β-sheet A is expanded to accommodate the insertion of s4A.[72] The introduction of a negative Asp or succinimide at position 191 could lower the activation energy to be overcome in the structural rearrangement required for s4A insertion, and so make it more likely that plasminogen activator inhibitor 1 will exist in this strand-inserted conformation. None of the other serpins has an Asn–Gly or a negatively charged residue at this position, and most have a lysine or glutamine, which could ion pair or hydrogen bond with the Glu or Asp at position 339.

Plasminogen activator inhibitor-1 has also been shown to exist in a nonnative conformation which functions as a proteinase substrate but not an inhibitor.[73] This form, as well as the latent form, are interconvertible with the inhibitor form, and although the substrate form has not been structurally characterized, it may be a partially inserted form like that found for antithrombin. If so, it supports the notion that the partially inserted serpin forms are noninhibitory substrates, fully inserted forms are neither substrates nor inhibitors, and that only the uninserted form is inhibitory.

α_1-Proteinase Inhibitor

The recombinant point mutants in s4A of α_1-proteinase inhibitor at positions P10 and P12[14] (Table I) further support the idea that s4A insertion is a necessary but not sufficient condition for serpin inhibitor activity. The P10 (Gly→Pro) mutant resembles the antithrombin Cambridge family of mutants in transforming the mutant from an inhibitor to a substrate. Similar arguments to those made above for the destabilization of s4A insertion in the P10 antithrombin mutants apply to the P10 α_1-proteinase inhibitor. This P10 mutant of α_1-proteinase inhibitor shows the stabilization to denaturation characteristic of the inhibitor serpins, thus resembling antithrombin Hamilton both in its shift from inhibitor to substrate and also in undergoing s4A strand

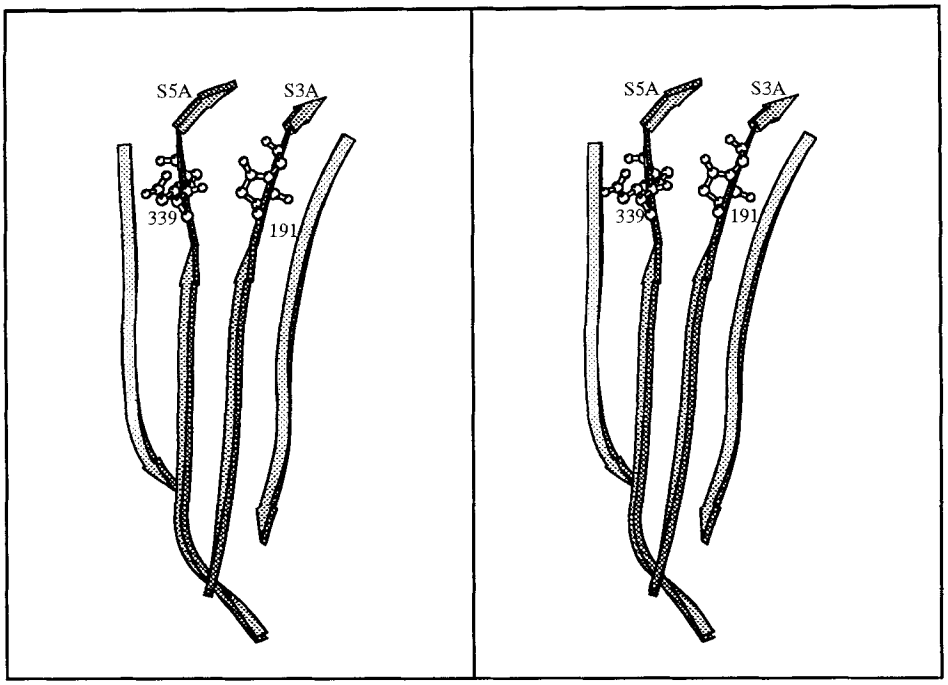


Fig. 3. Stereo view of model of intact plasminogen activator inhibitor 1 around insertion point of s4A showing the disruption of interstrand hydrogen bonds attendant on formation of a stable succinimide as a result of deamidation of Asn-191. The carboxylate sidechain of Glu339 is shown on strand s5A, in proximity to the succinimide at position 191 on s3A. Breakdown of the succinimide would yield a carboxylate at this position.

insertion, but also lacking inhibitor activity. The P12 (Ala→Thr) mutant is the same as antithrombin Hamilton, but in contrast to it retains inhibitor activity. Inspection of a model of this mutant shows that the mutant Thr-347 can hydrogen bond directly to Thr-345, in contrast to antithrombin Hamilton, which can only form a hydrogen bond to Ser-345 through an intervening water molecule. We suggest that this difference is critical to the rate of strand insertion, the capture of the water molecule retarding the rate of s4A insertion in antithrombin Hamilton, and thus exposing the acyl-linked proteinase to hydrolysis by solvent before formation of the protective, stable complex can occur.

A recent study of a recombinant α_1-proteinase inhibitor in which residue 345 was altered from a threonine to an arginine provides further support for the idea that the effects of variant residues in the s4A strand are on the kinetics of the inhibition reaction.[74] This mutant alters α_1-proteinase inhibitor to resemble ovalbumin in having a large, charged residue at residue 345, which projects into the interior of the molecule upon insertion of s4A. Studies of the T345R mutant show it to function primarily as a substrate, with stoichiometries of inhibition well above one for several target proteinases. Surprisingly, the cleaved mutant serpin undergoes the increase in stability indicative of strand insertion in the wild-type serpins, and also forms a very small amount of stable complex.

A preliminary model of this mutant points up a distinction between it and the comparable structure of ovalbumin with Arg-345 modeled from α_1-proteinase inhibitor in the strand-inserted conformation. In contrast to ovalbumin, Arg-345 of the T345R α_1-proteinase inhibitor mutant can be accommodated in the insertion pocket beneath β-sheet s4A. Furthermore, it can form a hydrogen bond between the N_ϵ of its guanidino group and the tyrosine hydroxyl of Tyr-244. It is also possible that the guanidino group may be further stabilized either by stacking between the phenyl rings of Phe-372 and Phe-252 or possibly forming weak hydrogen bonds to the π electrons of one of these rings. It seems unlikely that these stabilizing interactions could completely compensate for the burial of the charged guanidino group, but it may sufficiently increase the residence time of s4A in the inserted conformation for a small fraction of the molecules to form a highly stable complex, and thus result in low levels of inhibition.

DISCUSSION

The early steps of the mechanism by which serpins inhibit serine proteinases are likely to be similar to those for the large number of other substrate-like serine proteinase inhibitors[65] with the exception of the conformation of the reactive site chain. In the smaller proteinase inhibitors, the reactive site is in an exposed, preformed conformation which approaches optimum configuration for fit to

the serine proteinase active site. In the serpins, the reactive sites appear to be metastable and must undergo changes in structure if they are to maximize their binding interactions with the target proteinase. We suggest that the adoption of a helical conformation in intact serpins serves the function of protecting the reactive site from adventitious cleavage. Interaction of the proteinase active site with the serpin reactive site either occurs with a subpopulation of the serpin whose reactive site residues contiguous with P1 are in a β-sheet conformation or, more likely, binding induces the β-sheet conformation favored for the residues immediately around P1 of serine proteinase substrates and inhibitors.[13,17,49]

In contrast to the smaller proteinase inhibitors, serpins undergo several other steps subsequent to or simultaneous with formation of the initial complex, before reaching a final, highly stable, covalent serpin–proteinase complex. We propose that the first of these steps is the initiation of s4A insertion into β-sheet A at P15, which occurs as a result of the shift from α-helix to β-sheet in the reactive site segment upon binding to proteinase. The collapse of the reactive site α-helix into an extended β-sheet conformation on proteinase binding would alter the packing ratio of this segment, releasing over 15 Å of potential β-sheet, corresponding to 4 residues. This released polypeptide chain will be available for insertion at the hinge region, and permits insertion of P15 to P12 of s4A without requiring any major movement of the proteinase covalently linked and protected from solvent hydrolysis at the P1 end of the reactive site segment. The release of this segment of s4A disrupts a number of hydrogen-bond constraints on the chain, as documented in the comparison of the plakalbumin and ovalbumin structures (Wright and Scarsdale, in preparation). This relaxation, with the formation of a stable acyl linkage between serpin and proteinase, and the directionality on s4A movement imposed by the Glu-342–Lys-290 ion pair, permit reptation of the proteinase to the opposite end of the molecule, probably driven by cooperativity in the insertion of the remaining residues (P3–P11) of s4A into β-sheet A. This carries the proteinase to a new position at the opposite pole of the serpin molecule, where it is again protected from solvent attack on the acyl linkage to the proteinase (Fig. 4).

The formation of stable proteinase–serpin complex in this mechanism requires that the acyl-serine linkage between them be protected from solvent hydrolysis throughout. The migration of the proteinase from the reactive site end of the serpin to the opposite pole would have to be either very rapid or follow a pathway in which the intermolecular linkage is uninterruptedly protected from solvent. A rapid migration could occur between a proteinase binding site at the reactive site end to a stable binding site at the other pole, which would not be easily detectable kinetically. Alternatively, the proteinase may migrate slowly down the face of β-sheet A, following the insertion of s4A.

Patston et al.[22] determined the partition ratio between cleaved C1 inhibitor and kallikrein–C1 inhibitor complex, and found that higher temperature favors complex formation, consistent with a thermally driven reaction. The rate of formation of the stable end complex[75,76] may be limited by thermally driven fluctuations of the structure which open strands s3A and s5A and flex the hinge region at residues 192 and helices D and E, as suggested by Stein and Chothia.[72]

In either of these cases, the critical step which drives formation of the final stable complex is not the proteolytic cleavage of the serpin, but rather the full insertion of strand s4A which is coordinated with the structural dislocation documented by Stein and Chothia. This coordination of proteinase binding, strand insertion, secondary structure shifts, and attendant release of hydrogen-bond constraints on the proteinase-bound reactive site segment guarantees that complete proteinase migration is coupled to full strand insertion, thus minimizing exposure of the acyl linkage to hydrolysis by solvent.

Relationship of Mechanism to Structure

The mechanism which we propose here based on an analysis of model structures of several serpins leads us to conclude that the inhibitor function of serpins depends on the relative rates of specific steps in the chain insertion reaction. Perturbation of certain of these steps, as occurs with some point mutants in s4A, will alter the partitioning between proteinase–inhibitor complex and cleaved, substrate serpin. Oversized amino acid substitutions in s4A at positions P10, P12, and P14 would decrease the rate of s4A insertion and thereby increase the fraction of time in which the acyl linkage between serpin and proteinase is exposed to solvent.

The stabilization of serpin structure accompanying s4A insertion and observed by equilibrium physical measurements[45,46,77–79] is a necessary but not sufficient condition for inhibitor activity in serpins. Ovalbumin lacks inhibitor activity and is not stabilized in the cleaved form, consistent with the inability of s4A to insert due to oversized Arg-345. Antithrombin Hamilton has an Ala→Thr replacement at 347, which can insert into s4A as judged by both model building and equilibrium denaturation measurements, but lacks inhibitor activity and is a proteinase substrate. We attribute this to either premature partial s4A insertion due to excessive stability of the P14 to P10 insertion conformation, which renders the reactive site susceptible to proteinase without stable complex formation, or to a decrease in the rate of proteinase-bound s4A insertion. In plasminogen activator inhibitor 1, the lowered barrier to strand separation in β-sheet A increases the rate of

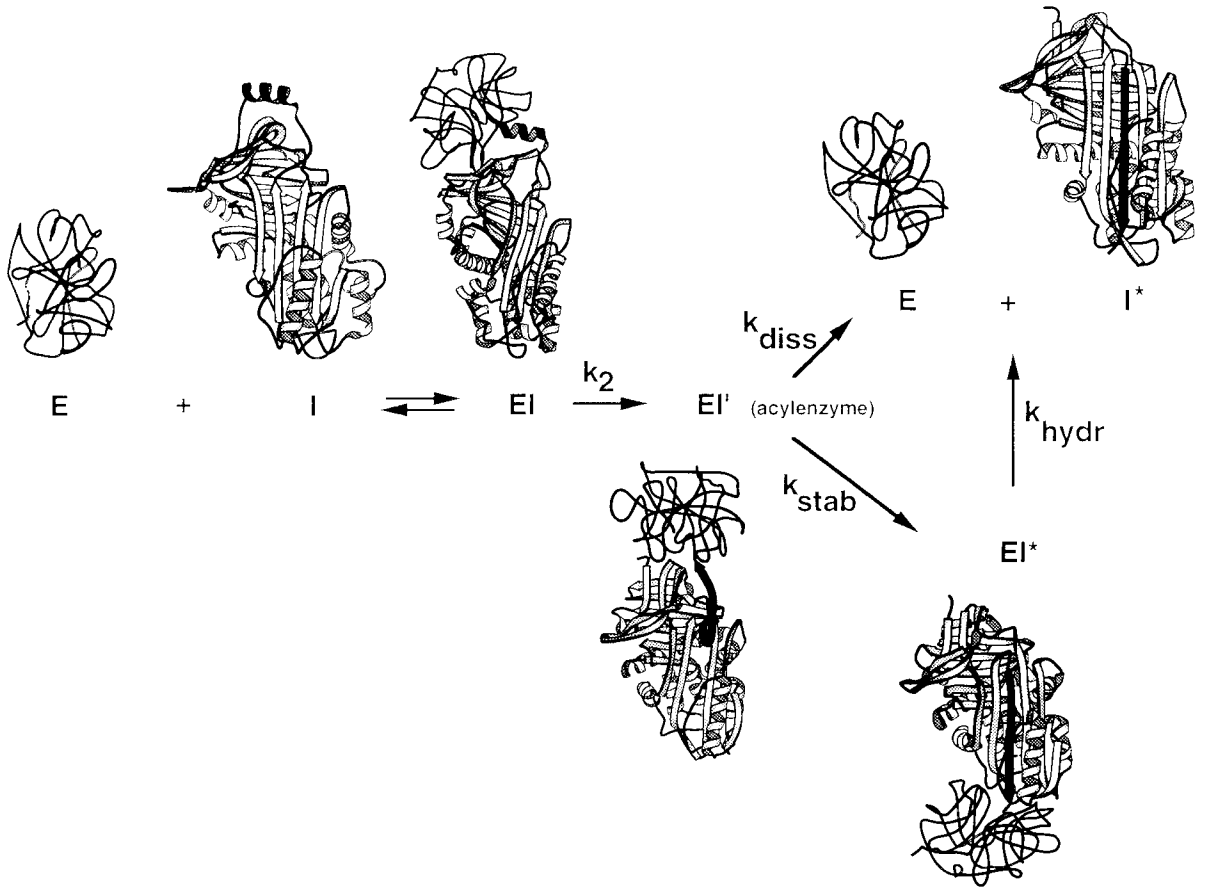


Fig. 4. Proposed mechanism for serpin inhibition of proteinases showing the structures of the serpin at each step of the reaction. Scheme is the same as Scheme 1, except that the tetrahedral intermediate (EI_{TI}), assumed to have the same structure as EI_M, has been omitted. Proteinase is shown as coil, serpin as ribbon and coil.

s4A insertion, and locks in the latent, strand inserted conformation. Finally, our interpretation of the case of antiplasmin Enschede represents an instance where we predict that complex formation occurs with full strand insertion, but the serpin–proteinase acyl bond is insufficiently protected from solvent at the end of the molecule distal to initial proteinase attack, resulting in deacyclation which gives rise to substrate but no inhibitor activity. Antiplasmin Enschede is not consistent with a requirement for preinsertion of s4A through residue P10, since the insertion mutation does not change the sequence at the residue mutated or immediately downstream of it.

Other Observations Relating to the Proposed Mechanism

Other observations are consistent with our proposal that the critical event in the formation of stable serpin–proteinase complexes is sequestration of the acylserpin–proteinase linkage by means of complete strand s4A insertion. Asakura et al.[80] isolated a monoclonal antibody which blocks formation of thrombin–antithrombin complex by triggering deacylation of an intermediate complex to yield cleaved antithrombin.[81] Stable thrombin–antithrombin complex is not susceptible to this effect of the antibody and the epitope of this antibody is residues 347–351[82] of strand s4A. We suggest that the observed deacylation is the result of failure of the antibody-bound strand s4A to insert, thus permitting the hydrolysis of the acylserpin–proteinase linkage.

Studies of binary complexes of serpins in which peptides corresponding to all or part of s4A are inserted into β-sheet A of intact serpin also bear on the role of s4A insertion in serpin function. Insertion of a peptide corresponding to wild type s4A of α_1-proteinase inhibitor abolishes trypsin–inhibitor activity, though no measurement of cleavage was made.[83] A similar experiment in which the s4A peptide of antithrombin was inserted into intact antithrombin converted the serpin into a substrate of thrombin without any evidence of complex formation.[49] This is consistent with hydrolysis of the uninserted, unprotected acylserpin–proteinase linkage. This work also showed that the binary peptide–antithrombin com-

plex was extremely stable, showing no unfolding transition below 82°, and indicating that insertion of full length s4A, even exogenously, leads to very stable conformations such as those observed for serpin–proteinase complexes.

A more extensive panel of α_1-proteinase inhibitor s4A peptides of different length was studied by Schulze et al.[48] for their effects on inhibitor activity. This work was interpreted to indicate the extent of preinsertion of the reactive site strand necessary to impose the conformation susceptible to proteinase attack, but other interpretations are possible. Peptides of length 12 or 14 when inserted into α_1-proteinase inhibitor render the serpin a proteinase substrate, an observation which we ascribe to the inability of the serpin s4A to initiate insertion and subsequently to fully insert and protect bound proteinase. Peptides truncated at the amino terminus to lengths of 11, 10, 9, or 8 residues retain some inhibitory activity (>90, 31, 30, and 10%, respectively), when measured after 5 min incubation for residual proteinase activity. The gradient of residual inhibitor activity from the 14 or 12 amino acid peptides through the 8mer does not follow expected stability of inserted peptide based on length. However, the peptides of length 11 and shorter would permit the initiation of s4A insertion at P15–P12, while priming insertion of the remainder of the strand by separating s3A and s5A. Initiation of insertion of s4A at P15–P12 could then make possible the displacement of the inserted exogenous peptide by the cooperative insertion of s4A. The gradient of inhibitor activity does correlate with the extent of s3A and s5A which would be separated and with the proximity of the inserted peptide amino terminus to the proposed initiation site for s4A insertion at P15–P12. Competition of the 11mer peptide and s4A for the P11 or P12 site, followed by rapid insertion of the remainder of s4A as a result of the prior separation of s3A and s5A, could confer higher inhibitor activity than for the shorter peptides, which do not impinge on the initiation site and which have incomplete extent of separation of s3A and s5A. It is also significant in this work that the amounts of stable complex formed in those binary complexes retaining inhibitor activity are low on SDS gels, indicating that the serpin–proteinase complex is either not formed or, if formed, is less stable or slower in reaching full stability than complexes in the absence of competing peptides.

The absence of natural serpin mutants at residues P8, P6, or P4 in s4A might be taken as evidence against the hypothesis of full strand insertion of s4A proposed here. The lack of mutants in this segment of s4A could be due to fewer constraints on the size and charge of s4A residues projecting below β-sheet A in this region. The recombinant P8 double mutant of α_1-proteinase inhibitor (Table I) created by site-directed mutagenesis[84] does not abolish inhibitor activity toward thrombin nor strand insertion measured by increased stability to denaturation. However, this Met→Glu change does not support the irrelevancy of the P8 position to serpin inhibition. A model of this mutant α_1-proteinase inhibitor (Fig. 5) shows that the inserted Glu can form hydrogen bonds with $N_\delta 1$ of histidine 334 and the $O_\gamma H$ of serine 56, and could thus exist in a stable inserted conformation.

The greater constraints on the size and charge of residues in the P10–P14 positions are imposed by the highly hydrophobic and tightly packed protein core underlying β-sheet A at those positions. The relatively greater effects of substitutions at these positions than at P8–P4 are also ascribable to their role in the initiation of s4A insertion, a step whose barrier may determine the rate of strand insertion and therby the partition of products between inhibited proteinase and cleaved serpin.

Recently, it has been shown that point mutations in s4A of plasminogen activator inhibitor 1, including one at P8, transform this serpin from an inhibitor to a substrate, thus implicating P8 in inhibitor function.[85] Also, the alteration of s4A residues inserting from P3–P8 in antiplasmin Enschede does not appear to compromise the strand-inserted conformation based on model building, but no data on the stability of this mutant to denaturation are available.

Large scale replacements of the reactive site loops of serpins also result in a pattern of inhibitor activity consistent with functional requirements of s4A beyond those of simple enzyme recognition and binding. Replacement of the P10–P10′ region of antithrombin by the corresponding 20 amino acids of prothrombin abolishes both antithrombin and Factor Xa activity.[86] Similarly, replacement of the P10–P10′ region of antichymotrypsin by the corresponding sequence from α_1-proteinase inhibitor resulted in a serpin which is a good substrate for human neutrophil elastase and an inhibitor of chymotrypsin with a stoichiometry of inhibition of 3.0.[51] Such a result is consistent with a failure to form a stable inserted s4A complex with elastase, bound through extensive interactions at the distal end of the serpin molecule. The fact that this mutant retains its chymotrypsin inhibitor activity suggests that insertion of the mutant s4A can occur, though its altered sequence raises the stoichiometry of inhibition. The complementarity of chymotrypsin with its cognate antichymotrypsin at the distal end of the molecule confers the stability which results in retained inhibitor activity.

Our proposed mechanism implies that the interaction between serpin and proteinase in the stable complex will also be a determinant of inhibitor vs. substrate activity, since this interface determines the final protection of the acyl linkage from solvent hydrolysis described by k_{hydr}. While cross-inhibitory activity of serpins does occur and can be directed by

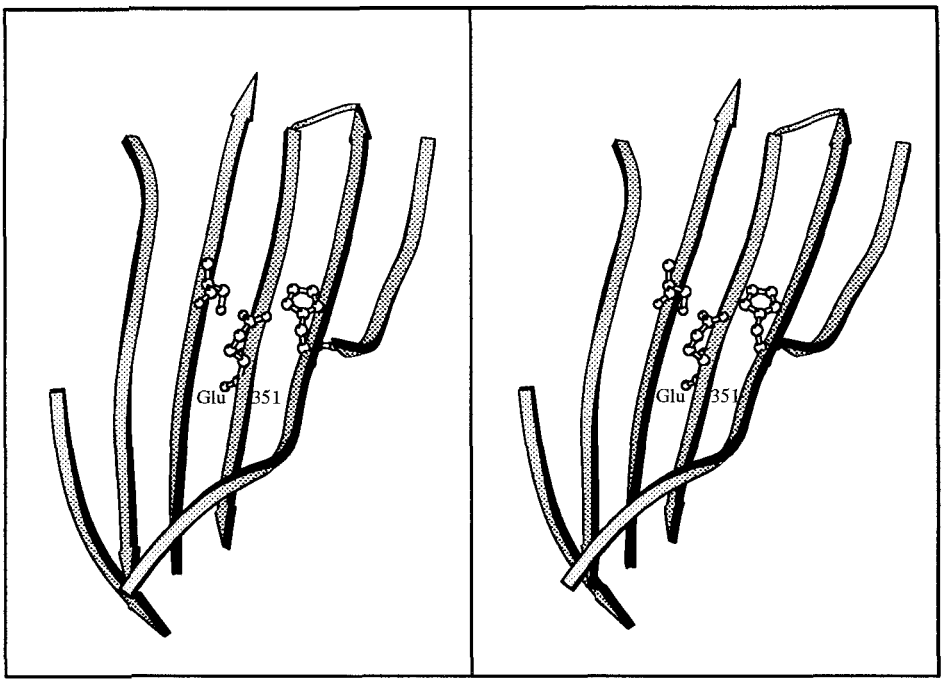


Fig. 5. Stereo view of model of P8 mutant (Met→Glu) in α_1-proteinase inhibitor, showing stabilization of Glu by hydrogen bonds to Ser-56 and His-334 in inserted conformation. View is from the interior of the molecule facing the underside of β-sheet sA.

the identity of the P1 residue, this residue alone is insufficient to confer inhibitor rather than substrate properties on a serpin. The myxoma virus serpin is an inhibitor of plasmin, urokinase, tissue plasminogen activator and C_{1s}, consistent with specificity for Arg at P1, but is a substrate of thrombin, trypsin, elastase, and other proteinases,[87] some of which are also specific for Arg at P1. This behavior would be expected if the contact surfaces between serpin and the latter proteinases are more permeable than the former to the infusion of solvent which hydrolyzes the acyl linkage between them. We predict that there will be other point mutants in serpins at the end of the molecule distal to the reactive site, which disrupt the interface and the stability of the terminal, highly stable proteinase–serpin complex.

The mechanism for serpin inhibition proposed here suggests multiple points at which serpin activity can be modulated. In addition to the strong specificity-determining P1 residue, sequence changes or mismatches between serpin and proteinase which alter the activation barrier to opening s3A and s5A to accept s4A or the final disposition of the acyl linkage between serpin and proteinase, can alter the partitioning between substrate and inhibitor complexes of serpins. We have not addressed the possible roles of heparin and of the unmodeled parts of plasmin, due to insufficient structural information, but they may also modulate the final proteinase–inhibitor complex stability.

Biological Implications

The mechanism of serpin inhibition proposed here is consistent with formation of stable proteinase–serpin complexes whose formation and stability are kinetically and thermodynamically controlled. The sequences of strands s3A, s4A, and s5A modulate serpin inhibitor formation and can alter the balance of substrate and inhibitor complex products of the reaction.

In vivo, serpin–proteinase complexes, and to a lesser extent cleaved serpins, are taken up by specific hepatocyte receptors.[85–95] However, there are also high concentrations of uncomplexed, cleaved, strand-inserted serpins in plasma, some of which, we propose, arise from slow deacylation of the proteinase from the serpin. We suggest that the nature of the processes which are regulated by serpins is such that transient and local, rather than long-term inhibition of proteinases is optimal. Complete removal of serpin–proteinase complexes, with feedback stimulation of serpin synthesis,[95] could deplete proteinase concentrations beyond that required for the primary biological response. Formation of serpin–proteinase complexes followed by slow modulated deacylation to free active proteinase and cleaved inactive serpin provides a mechanism for transient pulsed decrease of proteinase activity followed by gradual restoration, without rapid replenishment, of the serpin pool. The rates of complex breakdown, which we propose depend upon its struc-

ture, would thus govern the short-term level of proteinase activity.

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