Probing serpin reactive-loop conformations by proteolytic cleavage

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Several crystal structures of intact members of the serine proteinase inhibitor (or serpin) superfamily have recently been solved but the relationship of their reactive-loop conformations to those of circulating forms remains unclear. Here we examine reactive-loop conformational changes of anti-trypsin and anti-thrombin by using limited proteolysis and binary complex formation with synthetic homologous reactive-loop peptides. Proteolysis at the P10–P9, P8–P7 and P7–P6 of anti-trypsin was distorted by binary complex formation. The P1'–P2' bond in anti-thrombin was more accessible to proteolysis after binary complex formation, whereas cleavage at the P4–P3 bond was variably altered by synthetic peptide insertion. The proteolytic accessibility of the reactive-site P1–P1' bond of anti-trypsin and anti-thrombin binary complexes was identical with that of the

native form and no cleavage was observed in the hinge region (P15–P10) of either protein, whether native or as binary complexes. These results fit with the proposal that the hydrophobic reactive loop of serpins adopts a modified helical conformation in the circulation, with the hinge region being partly incorporated into the A β -pleated sheet. This loop can be displaced by peptides and induced to adopt a new conformation similar to the three-turn helix of ovalbumin. Both the native and binary complexed forms of anti-thrombin showed a greatly increased proteolytic sensitivity in the presence of heparin, indicating that heparin either induces a conformational change in the local structure of the helical reactive loop or facilitates the approximation of enzyme and inhibitor.

INTRODUCTION

The serpin superfamily of serine proteinase inhibitors comprises more than 80 homologous proteins that have been identified in animals, plants, insects and viruses (see [1–3] for review). Although the physiological importance of inhibitory serpins is well recognized, their mechanism of inhibition remains controversial [4–11]. This is primarily due to the different proposals, based on X-ray structural work, for the interaction of the serpin reactive loop with its target proteinase. Crystal structures have shown that serpins share a common molecular architecture centred on a large five-stranded A- β -sheet and a flexible reactive loop [12]; it is therefore likely that they have a common inhibitory mechanism. The plasticity of the reactive loop was illustrated by proteolytically modified structures in which cleavage of the scissile reactive bond, denoted as P1–P1' [13], resulted in a marked and irreversible conformational change with the N-terminal residues of the loop being completely incorporated into the middle of the A- β -sheet as strand 4A [14–18]. The intact serpin reactive loop can also be incorporated into the A-sheet to form a thermostable inactive molecule that shares immunological epitopes with the cleaved species [19]. This conformation is the structural basis for the latency of plasminogen activator inhibitor-1 [20] and we have recently demonstrated the existence of a similar conformation in anti-thrombin and anti-trypsin [21–23]. The crystal structure of the intact non-inhibitory serpin ovalbumin showed the reactive loop to be a fully exposed three-turn α -helix located above the plane of the molecule, leaving the A-sheet with one strand fewer [24]. Together these various conformations suggest that the serpin reactive loop can adopt several conformational rearrangements with the circulating active conformation likely to be an intermediate between the two inactive structural extremes: the exposed loop seen in ovalbumin and the buried loop as in the cleaved and latent species.

One proposal, based on the plasticity of the reactive loop, is that the inhibitory molecule adopts a canonical conformation similar to that reported in other classes of serine proteinase inhibitor [25]. To achieve this the small, non-polar residues of the hinge region of the loop (P15–P10) need to be incorporated into the gap between strands 3 and 5 of the A-sheet before reacting with their target proteinases, the 'pre-equilibrium conformational change model' [8,26]. Support for this model comes from the inaccessibility to proteolytic cleavage of the P15–P10 region of the loop [27] and also from the recently determined crystal structures of an anti-thrombin dimer [21,28]. These structures showed the hinge region of the reactive loop of one active intact molecule to be inserted into its A-sheet to form a partial canonical structure, whereas the other part of the loop was linked to a β -pleated sheet of a second, latent species.

An alternative proposal is that the reactive loop of the inhibitory serpins is induced to adopt a kinetically stable canonical conformation after docking with its cognate proteinase [6,29,30]. Support for this 'induced conformational change model' comes from the observation of partial inhibition and partial suicide substrate reaction for some serpin–proteinase interactions [7,31,32]. These observations indicate that proteinase molecules can escape from the trapping mechanism by cleaving the serpin reactive site before being locked into a stable complex by the insertion of hinge region. Point mutations within the hinge region introducing large or charged amino acid side chains would also block the locking mechanism and convert the serpin from inhibitor to substrate [6,11,33,34]. Moreover, ¹H NMR spectroscopy studies suggest that the serpin reactive loop is less mobile than previously thought [35] and recently a 'non-preinserted' distorted α -helical reactive loop containing the P3–P3' region of anti-trypsin was demonstrated in the crystal structure of intact active recombinant α_1 -anti-chymotrypsin [36].

At present we are unable to examine directly the confor-

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mational rearrangement of the serpin reactive loop during the inhibitory mechanism from the circulating native form to the final inactive cleaved state. It is possible, however, to adopt indirect approaches to probe the conformations of the reactive loop by studying its proteolytic vulnerability. The results are compatible with the proposal that a helical reactive loop, with defined mobility in the circulating native serpin, is necessary for protection against indiscriminate enzymic inactivation while allowing the molecule to adopt the range of conformations necessary for docking and locking with cognate proteinases.

MATERIALS AND METHODS

Materials

Human α -thrombin and human neutrophil elastase were gifts from Dr. Stuart Stone and Dr. David Bruce respectively, of the Department of Haematology, University of Cambridge, U.K. Heterogeneous porcine heparin was from Grampian Enzymes, Arthath, Aberdeen, U.K.; papaya proteinase IV was from CalBiochem-NovaBiochem Co., Nottingham, U.K.; bovine α -chymotrypsin, subtilisin, papain and thermolysin were from Sigma Chemical Co., Poole, Dorset, U.K. The synthetic peptides were prepared by the Department of Biochemistry, University of Cambridge, on an Applied Biosystems peptide synthesizer, (Foster City, CA, U.S.A.). The synthetic peptides were purified by reverse-phase HPLC on a C-18 column with a 30–50% (v/v) acetonitrile gradient in 50 mM ammonium acetate. The peptides were then freeze-dried and dissolved in 50 mM Tris, 50 mM NaCl, pH 7.4, before use.

Purification of native anti-thrombin and M anti-trypsin

Anti-thrombin was isolated from 2 litres of fresh frozen plasma by using heterogeneous heparin-Sepharose by the method of McKay [37]. Residual heparin was removed by loading the resulting anti-thrombin onto a Poros-Q (PerSeptive Biosystems, Cambridge, MA, U.S.A.) anion-exchange column in 20 mM Tris/HCl, pH 7.4, and eluting with a 0–1 M NaCl gradient. Anti-thrombin (10 mg/ml in 20 mM Tris/HCl, pH 8.0) was snap-frozen in liquid nitrogen and stored at -80°C until use. Anti-trypsin was purified from 200 ml fresh frozen plasma from an M homozygote by 50% and 75% (w/v) ammonium sulphate fractionations followed by thiol-exchange and Q-Sepharose chromatography (Pharmacia Biotechnology, St Albans, Herts., U.K.) as detailed previously [38]. The protein was snap-frozen and stored at 2 mg/ml in 50 mM Tris, 50 mM KCl, pH 7.4, with 0.1% (v/v) 2-mercaptoethanol. Anti-trypsin and anti-thrombin concentrations were determined by the A_{280} [39,40] and were more than 75% active against bovine α -chymotrypsin and human α -thrombin respectively [38,41].

PAGE

SDS/PAGE was performed with 10–20% (w/v) linear-gradient gels by the method of Laemmli [42] but using 2-amino-2-methyl-1,3-propanediol instead of Tris base. Non-denaturing PAGE was a modification of the method of Goldenberg [43] and carried out on 7.5–15% (w/v) linear gradient gels using 53 mM Tris, 68 mM glycine, pH 8.9, as the cathode buffer and 100 mM Tris/HCl, pH 7.8, as the anode buffer. In both gel systems 4–5 μg of protein was loaded in each lane.

Binary complex formation

Binary complexes were formed by incubating anti-trypsin or

anti-thrombin at a final concentration of 1 mg/ml in 50 mM Tris, 50 mM NaCl, pH 8.0, with a 100-fold molar excess of either a 13-mer peptide (Ac-Gly-Thr-Glu-Ala-Ala-Gly-Ala-Met-Phe-Leu-Glu-Ala-Ile-OH) corresponding to the P15–P3 segment of the reactive loop of anti-trypsin or a 12-mer peptide (Ac-Ser-Glu-Ala-Ala-Ala-Ser-Thr-Ala-Val-Val-Ile-Ala-OH) representing the sequence P14–P3 of the anti-thrombin reactive loop at 37°C for up to 24 h. Excess peptides were removed by concentration dialysis in 20 mM Tris/HCl, pH 7.4, in a Centricon-30 cell (Amicon Inc., Beverly, MA, U.S.A.). The presence of a binary complex was assessed by separating the proteins by 7.5–15% (w/v) non-denaturing PAGE.

Limited proteolysis of the reactive loop

In all experiments the final concentration of serpin in each cleavage reaction was 0.5 mg/ml. The assessment of the conformation of the reactive loop by limited proteolysis of native and binary complexed anti-trypsins with thermolysin was conducted at a 500:1 (by wt.) ratio of serpin to enzyme in metalloproteinase buffer (20 mM Tris, 2 mM CaCl_2 , pH 7.2) for 2 h at 37°C . Papain and papaya proteinase IV cleavages of anti-trypsin were performed at 75:1 and 50:1 (by wt.) ratios of inhibitor to proteinase respectively, in 30 mM potassium phosphate, 30 mM NaCl, pH 7.0, for up to 1 h at 37°C . Human neutrophil elastase was incubated with native and the two binary complexed forms of anti-thrombin at a 250:1 (by wt.) ratio of serpin to enzyme in 20 mM Tris/HCl, pH 8.0, for 6 h at 37°C . Anti-thrombin preincubated with heparin at 2.33:1 (by wt.) ratio for 10 min at 25°C was then cleaved by using 1000:1 (by wt.) ratio of anti-thrombin:elastase for 1 h at 37°C . Thermolysin cleavages of heparin-treated [2.33:1 (by wt.) ratio for 10 min at 25°C] and untreated anti-thrombins were performed at 400:1 (by wt.) ratio of inhibitor to proteinase in metalloproteinase buffer for 2 h at 37°C . All the reactions were terminated by the addition of SDS/polyacrylamide gel loading buffer [62.5 mM Tris, 3% (w/v) SDS, 10% (v/v) glycerol, pH 6.8] and heating at 95 – 100°C for 10 min before separating the proteins by SDS/PAGE. The relative rates of cleavage were determined by comparison of the amount of the cleaved material at each proteinase dilution. Each cleavage fragment of anti-thrombin was electrotransferred from gels onto ProBlott membrane (Applied Biosystems Ltd., Warrington, Cheshire, U.K.). For N-terminal sequencing, anti-trypsin samples were immediately snap-frozen in liquid nitrogen at the end of the cleavage reaction and freeze-dried. The N-terminal amino acid sequence of each cleavage fragment was then determined by the Department of Biochemistry, University of Cambridge, on an Applied Biosystems 477A protein sequencing system.

RESULTS

Formation of binary complexes

The 12-mer synthetic (P14–P3 of anti-thrombin) and 13-mer synthetic (P15–P3 of anti-trypsin) peptides were chosen because each represents the full length of reactive-loop peptide that inserts into the A-sheet of anti-thrombin and anti-trypsin respectively, as determined from crystallographic data of the cleaved structures. The peptides were incubated with each serpin in 100-fold molar excess and aliquots taken at different times to assess the rate of formation of serpin–peptide binary complexes. Both peptides induced the complete formation of homologous (serpin molecules containing synthetic peptide sequences derived from their own reactive loop) and heterologous (molecules with synthetic peptide sequences other than their own reactive loop)

Figure 1 Formation of homologous and heterologous binary complexes between serpins and synthetic peptides

A synthetic N_α-acetyl 12-mer reactive-loop peptide (P14–P3 of the anti-thrombin) and a synthetic N_α-acetyl 13-mer reactive-loop peptide (P15–P3 of the anti-trypsin) were incubated with native (N) anti-thrombin and anti-trypsin at a peptide-to-serpin molar ratio of 100:1 at 37 °C. The more anodal migration of the binary complex (BC) in this non-denaturing gradient PAGE (7.5–15% w/v acrylamide) is due to the acquisition of additional negative charge from the incorporated acetylated peptide. Lanes 1–5 represent aliquots of the incubation mixture of anti-trypsin with 12-mer peptide (heterologous binary complex) taken at 0, 3, 6, 12 and 24 h respectively. Lanes 6–10 were as for lanes 1–5, but with anti-trypsin incubated with the homologous 13-mer peptide. Lanes 11–15 correspond to samples of the incubation mixture of anti-thrombin with the 12-mer peptide (homologous binary complex) removed from the reaction at 0, 3, 6, 12 and 24 h respectively; lanes 16–20 represent samples of anti-thrombin incubated with the heterologous 13-mer peptide for the same periods.

binary complexes after incubation for 12 h at 37 °C (Figure 1). Previous studies showed that the formation of homologous anti-trypsin–peptide complexes was highly dependent on temperature and concentration [44–47]. We have confirmed this result by incubating the 12-mer peptide with anti-thrombin at a peptide-to-serpin ratio of 100:1 at 37 °C for 24 h. This resulted in 100 % binary complex formation, whereas incubation at 5:1 and 1:1 peptide-to-serpin ratios over the same period resulted in 70 % and 40 % binary complex formation respectively. Surprisingly, the rate of heterologous anti-trypsin–12-mer-peptide complex formation (Figure 1, lanes 2 and 3) was approximately twice that of the formation of the homologous anti-trypsin–13-mer-peptide complex (Figure 1, lanes 7 and 8). Similar results were observed in the formation of anti-thrombin heterologous complexes in which the rate was approximately four times that of the formation of homologous binary complexes (Figure 1, lanes 12, 13, 17 and 18). All the homologous and heterologous binary complexes showed greatly increased thermostability and behaved as substrates that were readily cleaved by their target proteinases (results not shown). Anti-thrombin binary complexes had a

Figure 3 Time course for the cleavage of native, heterologous and homologous binary complexed anti-trypsin after incubation with papain at a serpin-to-proteinase ratio of 75:1 (by wt.)

The proteins (4 µg per lane) were examined by SDS/PAGE (10–20% gel). Lane 1, molecular mass markers; lanes 2–5, samples removed from the cleavage reaction of native anti-trypsin at 0, 10, 20 and 30 min respectively. Lanes 6–9 represent cleavage of heterologous binary complex with the 12-mer anti-thrombin peptide after 0, 10, 20 and 30 min respectively. Lanes 10–13 represent cleavage of homologous binary complex with the 13-mer anti-trypsin peptide at the same time points. Abbreviations: N, native anti-trypsin; C, papain-cleaved anti-trypsin.

lower affinity for heparin than did native anti-thrombin, which is consistent with the observation of Björk et al. [29,47].

Limited proteolysis of anti-trypsin

Further experiments were conducted to observe the effect of binary complexes on the reactive-loop conformation by examining the rate of anti-trypsin cleavage by papain, thermolysin and papaya proteinase IV in a time-dependent manner. Cleaved materials from the various anti-trypsin forms were isolated and N-terminally sequenced, confirming the cleavage sites shown in Figure 2. SDS/PAGE demonstrated that the cleavage of native anti-trypsin by papain (Figure 3) and thermolysin (Figure 4) was approx. 20–50 % faster than that of the homologous and heterologous binary complexed forms. As described previously [27], papain cleaved primarily at the P7–P6 bond of native anti-trypsin, with a subsequent secondary cleavage at P1–P1'. However, in both binary complexed forms the cleavage pattern with papain was reversed, with the primary cleavage site being P1–P1'. Similar results were obtained for thermolysin cleavage of anti-trypsin. After incubation with enzyme to give incomplete di-

	P ₁₂	P ₁₁	P ₁₀	P ₉	P ₈	P ₇	P ₆	P ₅	P ₄	P ₃	P ₂	P ₁	P ₁ '	P ₂ '	P ₃ '
α ₁ -AT	A	A	G	A	M	F	L	E	A	I	P	M	S	I	P
			↑		↑	↑						↑			
			1		3	2, 3						2, 4			
ATIII	A	A	A	S	T	A	V	V	I	A	G	R	S	L	N
									↑			↑	↑		
									5			6	3		

Figure 2 Positions of proteolytic cleavage sites reported in this paper

1, papaya proteinase IV cleavage; 2, papain cleavage; 3, thermolysin cleavage; 4, bovine α-chymotrypsin cleavage; 5, human neutrophil elastase cleavage; 6, human α-thrombin cleavage. Abbreviations: α₁-AT, anti-trypsin; ATIII, anti-thrombin.

Figure 4 Time course for the cleavage of native, homologous and heterologous binary complexed anti-trypsin after incubation with thermolysin at a serpin-to-proteinase ratio of 500:1 (by wt.)

The proteins (4 μ g per lane) were examined by SDS/PAGE (10–20% gel). Lanes 1–8 represent samples removed from the cleavage reaction of native anti-trypsin at 0, 10, 20, 30, 45, 60, 90 and 120 min respectively. Lanes 9–16 represent cleavage of the homologous binary complex with the 13-mer anti-trypsin peptide over the same periods. Lanes 17–24 show cleavage of the heterologous binary complex with the 12-mer anti-thrombin peptide also at the same time points. Abbreviations: N, native anti-trypsin; C, thermolysin-cleaved anti-trypsin.

Figure 5 Proteolysis of anti-trypsin forms by papaya proteinase IV at a serpin-to-proteinase ratio of 50:1 (by wt.)

The proteins (4 μ g per lane) were examined by SDS/PAGE (10–20% gel). Lanes 1–7 represent samples removed from the cleavage reaction of native anti-trypsin at 0, 5, 10, 20, 30, 45 and 60 min respectively. Lanes 8–14 represent cleavage of homologous binary complex with the 13-mer anti-trypsin peptide over the same periods. Lanes 15–21 show cleavage of heterologous binary complex with the 12-mer anti-thrombin peptide also at the same time points. Abbreviations: N, native anti-trypsin; C, papaya-proteinase IV-cleaved anti-trypsin.

gestion of the native protein, N-terminal sequencing revealed that there was 40% cleavage at P7–P6, 20% cleavage at P8–P7 and 40% intact protein. In both binary complexed forms, the cleavage pattern was distorted after identical incubations with thermolysin. The homologous binary complex revealed a new cleavage site at N-terminal His¹⁶–Asp¹⁷ (37.5%) along with 37.5% cleavage at P7–P6 and 25% at P8–P7. The findings were more marked for the heterologous binary complex with increased accessibility of the P7–P6 bond (52%), no significant difference at P8–P7 (27%) and the remaining 21% showing no cleavage. There was variability in the final quality of anti-trypsin that was cleaved with thermolysin in a given period of time, which was dependent upon storage of the proteinase. Papaya proteinase IV, in comparison, cleaved at P10–P9 in the native molecule, but failed to cleave either of the binary complexes (Figure 5).

Figure 6 Comparison of the cleavage rates of native, homologous and heterologous binary complexed anti-thrombins by human neutrophil elastase

The proteins (4 μ g per lane) were examined by SDS/PAGE (10–20% gel). (Upper panel) Time course of cleavage of anti-thrombin by human neutrophil elastase at a serpin-to-proteinase ratio of 250:1 (by wt.). Lane 1, molecular mass markers; lanes 2–5, samples removed from the cleavage reaction of native anti-thrombin at 0, 1, 3 and 6 h respectively. Lanes 6–9, as for lanes 2–5 except that cleavage of homologous binary complex with the 12-mer anti-thrombin peptide was analysed. Lanes 10–13 correspond to the samples removed from the cleavage reaction of anti-thrombin heterologous binary complex with the 13-mer anti-trypsin peptide at 0, 1, 3 and 6 h respectively. (Lower panel) Three anti-thrombin forms were preincubated with heparin at 25 °C for 10 min before being subjected to time-course cleavage by human neutrophil elastase at a serpin-to-proteinase ratio of 1000:1 (by wt.). Lanes 1–5, samples taken from the cleavage reaction of native anti-thrombin at 0, 5, 15, 30 and 60 min respectively. Lanes 6–10 represent cleavage of homologous binary complex with the 12-mer anti-thrombin peptide over the same periods. Lanes 11–15, samples removed from the cleavage reaction of anti-thrombin heterologous binary complex with the 13-mer anti-trypsin peptide at 0, 5, 15, 30 and 60 min respectively. Abbreviations: N, native anti-thrombin; C, elastase-cleaved anti-thrombin.

Limited proteolysis of anti-thrombin

The rate of cleavage of three different forms of anti-thrombin by human neutrophil elastase and thermolysin was assessed. Human neutrophil elastase was found to cleave native, homologous and heterologous binary complexes of anti-thrombin at the P4–P3 peptide bond. SDS/PAGE showed that the cleavage rate was much faster for the heterologous binary complex containing the 13-mer anti-trypsin peptide than for native anti-thrombin, but surprisingly the homologous binary complex containing the 12-mer anti-thrombin peptide became almost inaccessible to proteolysis (Figure 6, upper panel). Thermolysin cleaved the P1'–P2' bond of both binary complexed forms of anti-thrombin at a rate 50 times that of the native protein (Figure 7, upper panel). Interestingly, incubation of these anti-thrombin conformations with heparin at 25 °C for 10 min markedly increased the rate of cleavage of the P4–P3 and P1'–P2' bonds in native and the two binary complexed forms of anti-thrombin; all these species became extremely susceptible to attack by human neutrophil

Figure 7 Time course for the cleavage of native, homologous and heterologous binary complexed anti-thrombin after incubation with thermolysin at a serpin-to-proteinase ratio of 400:1 (w:w)

The proteins (4 μ g per lane) were examined by SDS/PAGE (10–20% gel). (Upper panel) Lane 1, molecular mass markers; lanes 2–8 samples removed from the cleavage reaction of native anti-thrombin at 0, 5, 15, 30, 60, 90 and 120 min respectively. Lanes 9–15 and lanes 16–22, as for lanes 2–8 except that cleavage of homologous anti-thrombin/12-mer peptide and heterologous anti-thrombin/13-mer peptide binary complexes respectively were studied. (Lower panel) All anti-thrombin forms were preincubated with heparin at 25 $^{\circ}$ C for 10 min before being subjected to limited proteolysis. Lane 1, molecular mass markers; lanes 2–8, samples taken from the cleavage reaction of native anti-thrombin at 0, 5, 15, 30, 60, 90 and 120 min respectively. Lanes 9–15, as for lanes 2–8 except that cleavage of homologous anti-thrombin/12-mer peptide complex was studied. Lanes 16–22, samples removed from the cleavage reaction of heterologous anti-thrombin/13-mer peptide complex at 0, 5, 15, 30, 60, 90 and 120 min respectively. Abbreviations: N, native anti-thrombin; C, thermolysin-cleaved anti-thrombin.

elastase and thermolysin (Figure 6, lower panel, and Figure 7, lower panel).

DISCUSSION

The serpins control the proteolytic pathways of coagulation, fibrinolysis and inflammation. To achieve their diverse roles they have evolved a complex structure based on a dominant five-membered A-sheet that supports a flexible reactive loop. The flexibility of the loop allows the molecule to adopt a range of conformations from the exposed helix of ovalbumin to the partial canonical structure of anti-thrombin and the fully inserted loop seen in the cleaved and latent states. We show here that the loops of anti-trypsin and anti-thrombin are induced to adopt differing conformations after the formation of binary complexes with synthetic peptides corresponding to the reactive-loop sequences.

The insertion of homologous reactive-loop peptides into both anti-trypsin and anti-thrombin supports previous observations that synthetic peptides are likely to incorporate as a sixth strand in the middle of the A-sheet [29,44–47]. Similarly the insertion of the anti-trypsin loop peptide into anti-thrombin and the anti-thrombin loop peptide into anti-trypsin also rendered the protein thermostable, a substrate rather than an inhibitor, and in the case of anti-thrombin reduced the heparin binding affinity to a value similar to that of the cleaved state.

Limited proteolysis studies of native anti-trypsin corroborate

the observations of Mast et al. [27] and support their deduction that the reactive loop is partly inserted into the A-sheet in the circulating conformation. However, the alteration in cleavage pattern after binary complex formation (Figures 3–5) is at variance with these authors' findings and suggests that binary complex formation does distort the hinge region and perturb the overall conformation of the loop as described previously [38]. The inserted peptides expel the loop from the A-sheet abolishing the accessibility of the P10–P9 bond and distorting the cleavage pattern with both papain and thermolysin. If the reactive loop of the active inhibitory serpins were identical with the three-turn helix of ovalbumin, one would expect the P1–P1', P5–P4, and possibly P8–P7 or P9–P8 peptide bonds to lie at the apex of the α -helical turns and so be accessible for cleavage (Figure 8A). The enzymic cleavage positions in the reactive loop of anti-trypsin, anti-thrombin and nine other active serpins reveal preferential cleavage sites at the P1–P1', P5–P4, and possibly P7–P6 and P10–P9 bonds (Figure 8B), consistent with the loop conformation of inhibitory serpins *in vivo* adopting a helix-like structure, but this helical conformation is not the same as the three-turn α -helix of ovalbumin. The crystal structure of recombinant α_1 -anti-chymotrypsin (Figure 8C) reveals an exposed distorted helical reactive loop containing one turn of a 3_{10} -helix (P10–P7), a β -turn (P6–P3), a novel 1.5-turn (P3–P3') and a type I β -turn (P2'–P5'). The crystal analysis of an anti-thrombin dimer reveals the helicity in the reactive loop of active molecule to be lost, which may result from distortion of the loop on binding to a

Figure 8 Schematic representation of the reactive-loop conformations of inhibitory serpins

(A) The non-inhibitory ovalbumin [24] showing a fully exposed three-turn α -helical reactive loop (black). (B) The cumulative plot of 67 proteolytic cleavages of peptide bonds between P15–P5' within the reactive loop of 11 different serpins showing that the frequency of cleavages is altered at different positions. (C) The distorted helical reactive loop (black) in the intact active recombinant mutant of α_1 -anti-chymotrypsin containing the P3–P3' region of anti-trypsin [36]. (D) The active molecule obtained from the anti-thrombin dimer, demonstrating a partial insertion of the reactive loop into the A-sheet [21]. (E) The proposed circulating form of inhibitory serpins shown by a half open A-sheet (white) allowing partial insertion of the reactive loop (black), which contains a two-turn helical structure. (F) The proposed conformation of anti-thrombin binary complex showing the reactive loop expelled by the insertion of a 12-mer peptide into the A-sheet to form a 3-turn helix similar to that of ovalbumin. (G) The proposed conformation of anti-thrombin binary complex showing that the incorporated 13-mer peptide containing one more residue than the 12-mer peptide would alter the peptide bond positions in the helical reactive loop, changing their accessibility to proteolysis.

second anti-thrombin molecule (Figure 8D). These observations, taken together with the absence of cleavage in the serpin hinge region, are compatible with a conformation of the serpin reactive loop in circulating form as a helix-like structure, such as a two-turn helix with unfolding of the third turn and partial insertion into the A- β -sheet (Figure 8E). The different cleavage patterns of papain, thermolysin and papaya proteinase IV with anti-trypsin binary complexes demonstrate the reduced accessibility of the P7–P6 and P10–P9 bonds, indicating that these positions are likely to be at the base of the second and first turn of the three-turn helical loop. In the transition from the two-turn loop to the three-turn conformation, the serpin hinge region needs to be expelled from the A-sheet to form the stalk of the newly formed helix, and as a consequence this region in binary complexed species predictably becomes accessible for cleavage. However, the binary complexes of anti-trypsin and anti-thrombin could not be cleaved at this hinge region with subtilisin, thermolysin, papain, *Staphylococcus aureus* V8 protease and papaya proteinase IV (results not shown), indicating either that these enzymes do not recognize this exposed domain or that the

peptides in the binary complexes alter the reactive-loop conformation, not by displacing the hinge region but by changing the structure of the A-sheet to which the loop is anchored.

The results of peptide insertions into anti-thrombin molecules are more striking and provide evidence that binary complex formation does not always render the molecule less susceptible to proteolytic attack (Figure 6, upper panel). Moreover, the peptide bonds in the helical reactive loop of binary complexes vary their positions after insertion of peptides of different lengths into the A-sheet. This is again consistent with the conclusion that the reactive loop of circulating serpins is likely to be a two-turn helix. These data, together with the various proteolytic patterns of the three different forms of anti-trypsin, fit with a model of the serpin reactive loop in the active and binary complexed conformations, as in Figures 8E–8G.

Unlike most serpins, anti-thrombin has a distinct heparin-binding domain, which allows heparin to greatly enhance anti-thrombin inhibition of thrombin (for reviews see [48,49]). Previous studies have suggested that the rapid inactivation of native anti-thrombin by human neutrophil elastase requires the presence

of heparin binding specifically to anti-thrombin [50]. We have confirmed this result by showing that not only the native form but also the two binary complexed forms of anti-thrombin exhibit their extreme susceptibility to proteolysis after binding to heparin (Figure 6, lower panel, and Figure 7, lower panel). This may reflect a heparin-induced conformational transition in anti-thrombin or the approximation effect of enzyme and inhibitor described by Jordan et al. [51]. Peptide insertion into anti-thrombin is hindered in the presence of heparin [47], in keeping with a conformational change that involves a closure of the A-sheet. A current proposal [52] is that this conformational change results in an expulsion of the reactive loop from the partly inserted form seen in anti-thrombin in the absence of heparin (Figure 8E) to the active semihelical form as in α_1 -anti-chymotrypsin (Figure 8C). This proposal is supported by the finding here that the proteolytic susceptibility of the binary complexed anti-thrombin mimics that of heparin activated anti-thrombin (Figure 7). What is more difficult to explain is the difference in proteolytic cleavage between the 12-mer and 13-mer binary complexes (Figure 6, upper panel), an effect that is abolished when the binary complexes are preincubated with heparin (Figure 6, lower panel).

The inference from the findings in general is that in the circulation, the serpin reactive loop has a helical structure with the hinge region partly incorporated into the A-sheet. The hinge region can be displaced by synthetic peptides to undergo a reactive-loop conformational change, most probably into a three-turn helix as seen in ovalbumin. The defined mobility of the helical reactive loop in circulating serpins would therefore favour the 'induced conformational change model', as the 'pre-equilibrium conformational change model' assumes a very floppy reactive loop incessantly adopting different conformations until one of them binds to form a tight complex with the cognate proteinase. However, these data do not completely negate the latter model because the insertion of a synthetic peptide might involve a modification of the whole serpin molecule, as implied by a newly found cleavage site for thermolysin at the N-terminus of the anti-trypsin homologous binary complex.

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