An examination of the inhibitory mechanism of serpins by analysing the interaction of trypsin and chymotrypsin with α_2 -antiplasmin

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Human α_2 -antiplasmin (α_2 -AP) has previously been shown to possess overlapping inhibitory sites for trypsin and chymotrypsin [Potempa, Shieh and Travis (1988) *Science* **241**, 699–700]. Since this is currently unique among active-site-directed inhibitors of proteinases, and difficult to explain in terms of accepted inhibitory mechanisms, we re-examined the claim. Initial characterization of purified α_2 -AP revealed an additional 12 residues preceding the published N-terminus, prompting us to revise the previous numbering. We found that trypsin caused cleavage of the Arg³⁷⁶-Met³⁷⁷ bond in the reactive-site loop of the inhibitor, whereas chymotrypsin caused cleavage at two sites in approx. equal amounts at 37 °C: Met³⁷⁴-Ser³⁷⁵ (site 1) and Met³⁷⁷-Ser³⁷⁸ (site 2). At 0 °C α_2 -AP became a more efficient inhibitor of chymotrypsin,

and the proportion of cleavage at site 1 declined, indicating that chymotrypsin prefers to react with site 2 at 0 °C. Inhibitors of the α_2 -AP type are inactivated when cleaved in their reactive-site loops by proteinases that they do not inhibit, so we conclude that site 1 is treated as a substrate by chymotrypsin. Site 2 is the inhibitory site for chymotrypsin. We confirm that α_2 -AP does indeed have overlapping reactive sites for trypsin and chymotrypsin, and since the locations of chymotrypsin-interaction sites vary with temperature, we suggest that α_2 -AP cannot have rigid reactive-site geometry. More likely, it has a mobile reactive-site loop of the type that has been recently demonstrated for eglin C.

INTRODUCTION

Proteolytic enzymes are ubiquitous. Of the four known catalytic classes, the subgroup of serine proteinases known as the chymotrypsin superfamily contains the most members in mammals (Barrett and McDonald, 1980). Individual members have widely differing substrate specificities and catalytic efficiencies, and may digest food or phagocytosed pathogens, or partake in most of the haemostatic mechanisms of the body. To prevent adventitious proteolysis of host proteins, a number of protein inhibitors of these proteinases have evolved. Most of the inhibitors are members of the serpin superfamily (Carrell and Boswell, 1986), and quite often one finds that a particular serpin targets a particular proteinase for inhibition, to achieve regulation of that particular proteolytic activity.

Members of serine proteinase inhibitor superfamilies known as kunins and kazals, which are not related to serpins, are found in mammals. The classification of proteinase inhibitor families originates from that of Laskowski and Kato (1980); so that 'kunins' are members related to the pancreatic trypsin inhibitor (Kunitz) family, and 'kazals' are related to the pancreatic secretory trypsin inhibitor (Kazal) family. Much is known of the mechanism of inhibition of serine proteinases by kunins and kazals (for reviews see Laskowski and Kato, 1980; Read and James, 1986), though little is known of their biological roles, with the notable exception of the kazal pancreatic secretory trypsin inhibitor which prevents premature activation of pancreatic proteinase zymogens. Paradoxically, the biological roles of a number of serpins are well documented (Travis and Salvesen, 1983), though their mechanism of inhibition is still a mystery (Huber and Carrell, 1989; Bode and Huber, 1991). This is largely due to the lack of success in obtaining crystals of serpin proteinase inhibitors for high-resolution diffraction studies.

The serpins α_1 -proteinase inhibitor (α_1 -PI), antithrombin III, plasminogen activator inhibitor 1, and α_2 -antiplasmin (α_2 -AP), which are respectively responsible for regulating neutrophil elastase, thrombin, tissue plasminogen activator, and plasmin, have been used by different research groups to investigate the inhibitory mechanism. The use of different proteins is based on the reasonable, though unproven, assumption that they are likely to share a common inhibitory mechanism since they are homologous and sometimes inhibit the same proteinases (Travis et al. 1990; Bode and Huber, 1991; Longstaff and Gaffney, 1991; Mast et al., 1991a). Our group's strategy for investigating the inhibitory mechanism is based on the premise that serpins are mechanistically similar to kunins and kazals, though they have evolved a distinct mechanism for displaying their proteinase-inhibitory site (Mast et al., 1991a, 1992). If this is the case, then deviations of serpin inhibitory activities from the 'standard-mechanism' kunins and kazals should give valuable hints in elucidating the mechanism.

The standard mechanism describes a process whereby protein inhibitors bind tightly to serine proteinases, so that the enzymic activity is blocked. Inhibition results from the association between the substrate-binding sites of the enzyme and the inhibitor's reactive-site loop (RSL). In inhibitors that follow the standard mechanism the RSL has a conformation that is complementary to the enzyme's substrate-binding sites. Small adjustments may occur during complex formation, but the RSLs of proteinase-complexed inhibitors from divergent families are all superimposable in conformation. The primary binding site of standard-mechanism inhibitors (known as P₁) is identical in different proteinase complexes; to bind a proteinase at a different site within the same RSL would be a deviation from the mechanism. Such a deviation is seen in α_2 -AP, the only proteinase inhibitor we know of that presents separate primary binding sites

Abbreviations used: α_1 -PI, α_1 -proteinase inhibitor; α_2 -AP, α_2 -antiplasmin; DCI, 3,4-dichloroisocoumarin; RSL, reactive-site loop; TFA, trifluoroacetic acid.

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for trypsin and chymotrypsin (Potempa et al., 1988). Our report investigates this apparent anomaly to the standard mechanism of inhibition.

MATERIALS AND METHODS

Materials

The chromogenic substrate for bovine trypsin (H-D-Pro-Phe-Arg-*p*-nitroanilide) was from Helena Laboratories, and the substrate for bovine chymotrypsin (*N*-succinyl-Gly-Gly-Phe-*p*-nitroanilide) was from Bachem Inc. Bovine trypsin and chymostatin were from Sigma. The general serine proteinase inhibitor 3,4-dichloroisocoumarin (DCI) was from Boehringer-Mannheim. Bovine α -chymotrypsin was from Worthington. Human α_2 -AP was purified by affinity chromatography on a Sepharose-plasminogen column as described by Wiman (1980). Recombinant human α_1 -PI was a gift from Dr. Hans-Peter Schnebli, Ciba-Geigy AG, Basel, Switzerland. Human neutrophil elastase was a gift from Drs. Wieslaw Watorek and James Travis, University of Georgia, Athens, GA, U.S.A.

Identification of cleavage sites in the RSL of α_2 -AP

α_2 -AP (66 μ g) was incubated with trypsin (12 μ g of total protein) or α -chymotrypsin (12 μ g of total protein) at 25 °C. After 1 min the reaction mixture was acidified by the addition of trifluoroacetic acid (TFA) to 5% (v/v). The C-terminal peptides released from α_2 -AP following the acid denaturation were purified by reverse-phase h.p.l.c. employing a linear gradient from 0 to 60% acetonitrile in 0.1% TFA. This was performed using a 2 mm $\times$ 220 mm Aquapore RP-300 column (Applied Biosystems) connected to an Applied Biosystems 130A h.p.l.c. system. The purified peptides were analysed and quantified by Edman degradation, and the size of the peptides was estimated by m.s.

Amino-acid-sequence analysis

Automated Edman degradation was carried out in an Applied Biosystems 477A sequencer with on-line phenylthiohydantoin analysis using an Applied Biosystems 120A h.p.l.c. system. The samples were applied to Porton peptide or protein sample support disks employing the modified cycles PI-BGN and PI-1 recommended by Porton instruments. Some samples were transferred from PAGE to poly(vinylidene difluoride) membranes (Matsudaira, 1987) before sequencing.

M.s. analysis

Aliquots of the C-terminal peptides were analysed by time of flight m.s. The mass spectrum was recorded using nitrocellulose targets in an Applied Biosystems BioIon 20 plasma desorption instrument. The spectrum was collected for 2–15 h. Further details of the instrumentation and spectral analysis have been described elsewhere (Sundqvist et al., 1984).

PAGE

SDS/PAGE was performed in 5–15% (w/v) acrylamide gradient gels (10 cm $\times$ 10 cm $\times$ 1.5 mm) using the glycine/2-amino-2-methyl-1,3-propanediol/HCl system described by Bury (1981). Non-denaturing PAGE was carried out in the same gel system

but with omission of SDS from the buffers. SDS/PAGE standard proteins were obtained from Bio-Rad, to which we added rat α_1 -inhibitor₃ (which contains components of 120 and 190 kDa).

α_2 -AP determination

The concentration of α_2 -AP was determined by amino-acid analysis. Samples of α_2 -AP were dialysed into water and their absorbance at 280 nm determined. A portion was dried, hydrolysed for 24 h in 6 M HCl, and applied to a Beckman 6300 amino-acid analyser. We used the residue composition to determine an absorbance coefficient, $A_{280}^{1\%, 1\text{cm}}$ 13.0, approx. twice the reported value of Lijnen and Collen (1986).

Active-site standardization of proteins

The active concentration of trypsin was determined as described previously (Chase and Shaw, 1967). This trypsin was used to standardize a preparation of α_1 -PI. The active concentration of human neutrophil elastase and bovine chymotrypsin was determined by titration with active-site-standardized human α_1 -PI as described by Salvesen and Nagase (1989).

Titration of α_2 -AP with trypsin and chymotrypsin

The concentration of α_2 -AP was standardized with respect to trypsin and chymotrypsin by titration at relatively high concentration to force rapid complex formation, followed by rapid dilution and assay. Active-site-standardized samples of trypsin or chymotrypsin were allowed to react with α_2 -AP in 160 μ l of 50 mM Hepes buffer, pH 7.4, containing 100 mM NaCl and 0.05% Nonidet P-40 (assay buffer), for 15 min at 0 °C or 37 °C. Reactant concentrations were 4×10^{-9} M for trypsin and from 0 to 1.5×10^{-8} M for α_2 -AP, or 8×10^{-8} M for chymotrypsin and from 0 to 2.5×10^{-7} M for α_2 -AP. An appropriate substrate (40 μ l of a 2 mM solution) was added, and substrate hydrolysis followed at 37 °C using a Molecular Devices V_{max} plate reader operating in the kinetic mode.

RESULTS

Characterization of α_2 -AP

Edman degradation of samples of purified α_2 -AP revealed two prominent N-terminal sequences (Figure 1). Quantification of phenylthiohydantoin derivatives in the first two cycles indicated that the N-terminal-extended species accounted for from one third to one half of the total α_2 -AP. This finding is in disagreement with previous reports that found only the downstream sequence (see the Discussion) in plasma-derived α_2 -AP, though the upstream sequence was seen in recombinant α_2 -AP (Sumi et al.,

Figure 1 Edman degradation of purified plasma α_2 -AP indicates the presence of two species

One of these lacked residues 1–12, presumably resulting from cleavage of the Pro-Asn bond during transport or purification of the protein. The relative amounts of each species was variable, and the numbers indicate the range of each species observed in several preparations. Asn¹³ is the N-terminus reported by previous authors.

Figure 2 Trypsin (a) and chymotrypsin (b) were titrated with α_2 -AP at 0 °C (open circles) or 37 °C (filled circles)

Each datum point is the residual enzymic activity in the presence of the corresponding molar ratio of inhibitor:enzyme. Trypsin apparently requires one molar equivalent for inhibition, irrespective of temperature, whereas chymotrypsin apparently requires 0.9 molar equivalents at 37 °C and 1.6 molar equivalents at 0 °C.

1989). The two forms were not readily separable by conventional isolation techniques. A small amount of sequence, starting at residue Met³⁷⁷, was observed, indicating that about 10% of the freshly purified protein had been cleaved in the RSL.

Interaction between α_2 -AP and trypsin or chymotrypsin

Since the binding of chymotrypsin by α_2 -AP is relatively weak (K_i 0.1 nM), it was necessary to conduct the titrations with this enzyme at a relatively high reagent concentration and to utilize a relatively insensitive substrate. This contrasts with inhibition of trypsin by α_2 -AP which is several orders of magnitude tighter (results from Longstaff and Gaffney, 1991). Titration of trypsin or chymotrypsin with α_2 -AP resulted in a linear response typical of tight inhibition (see Figure 2 for representative titration curves).

Samples were run in SDS/PAGE and non-denaturing PAGE systems to resolve the different molecular forms after titration of α_2 -AP by trypsin or chymotrypsin (Figure 3). In these titrations the α_2 -AP concentration was kept constant and the enzyme concentration varied from 0 to 1.2 molar equivalents. Titration with trypsin (Figure 3a) resulted in formation of a small amount of SDS-stable complex, and substantial amounts of RSL-modified material.

[The nomenclature used to describe serpin forms follows that of Laskowski and Kato (1980), wherein 'virgin' describes a native inhibitor that is fully active and has not reacted with a proteinase, and 'modified' describes an inhibitor that has been proteolysed somewhere in the RSL.]

The RSL-modified material migrated as two components: a C-terminal peptide of about 10 kDa and the remainder migrating at around 55 kDa (Figure 3a). SDS-denaturation of the chymotrypsin/ α_2 -AP reaction mixtures resulted in substantially more modified material and less SDS-resistant complex than denaturation of the trypsin/ α_2 -AP mixtures (Figure 3b). We found the proportions of virgin, modified and proteinase-complexed α_2 -AP, seen in SDS/PAGE, to be highly reproducible in different preparations of the inhibitor, in contrast with Longstaff and Gaffney (1991) who reported variable proportions of these. However, it was important that chymostatin or DCI be

included in the mixtures just before electrophoresis to prevent degradation by released proteinase.

The formation of α_2 -AP-trypsin complexes is readily detectable in non-denaturing PAGE. α_2 -AP/chymotrypsin complexes are seen also in non-denaturing PAGE, though the extent of complex formation is never as complete as with trypsin [Figures 3(c) and 3(d)]. Trypsin/ α_2 -AP and chymotrypsin/ α_2 -AP complexes break down to give substantial amounts of modified α_2 -AP when run in SDS/PAGE [compare lanes 6 in Figures 3(a) and 3(c)]. Secondary modifications that affect the migration of the chymotrypsin-generated components are apparent when the chymotrypsin/ α_2 -AP ratio exceeds 0.2 (Figure 3d, lanes 3–7). These modifications were determined by Edman degradation of each of the components following their transfer from native gels to poly(vinylidene difluoride) membranes. Both bands in lanes 3–7 of Figure 3(d) contained the sequence Lys-Ser-Pro-Pro-Gly-Val and the sequence Ser-Leu-Ser-Ser-Phe, indicating hydrolysis of the Leu³⁶-Lys³⁷ bond near the N-terminus, and the Met³⁷⁷-Ser³⁷⁸ RSL peptide bond of α_2 -AP by chymotrypsin (Figure 4). This is in marked contrast with trypsin/ α_2 -AP reactions where no additional RSL cleavage was detected in the lower bands seen in non-denaturing PAGE beyond the small amount present in the starting material (Figure 3c, lanes 2–5). Trypsin and chymotrypsin components were detected in the upper bands of lanes 3–7 of Figures 3(c) and 3(d), confirming that these represent proteinase-inhibitor complexes. RSL cleavage observed following Edman degradation of the complex bands from the non-denaturing gels is not a reliable indicator of cleavage having occurred under native conditions. This is because the process of Edman degradation denatures proteins and can force this cleavage to occur.

Occasionally, at a molar ratio of chymotrypsin of 0.2, hydrolysis of the Leu²⁰-Thr²¹ bond of α_2 -AP preceded cleavage of the Leu³⁶-Lys³⁷ bond. We note that N-terminal trimming of α_2 -AP by chymotrypsin and trypsin occurred under non-denaturing conditions in the presence of excess α_2 -AP. We conclude that the N-terminal region of α_2 -AP can be cleaved by uncomplexed proteinases, and that the region around Leu³⁶-Lys³⁷ represents an unusually sensitive site for proteolysis. Substantial amounts of the inhibitor are inactivated by chymotryptic cleavage in the RSL during reaction, without complex formation. This inactivation is not seen with trypsin.

Location of RSL cleavage sites

The chymotrypsin titration showed significant temperature dependence, such that α_2 -AP appeared to be a worse inhibitor of this enzyme at elevated temperatures. This suggests that competition exists for chymotrypsin; it can either inactivate α_2 -AP, or it can be inhibited by α_2 -AP. The basis for this observation was explored.

Trypsin or chymotrypsin was allowed to react with a 2-fold molar excess of α_2 -AP at 0 °C and 37 °C, the reaction was terminated by TFA and samples were run in h.p.l.c. as described in the Materials and methods section. The trypsin reaction contained a major peptide peak eluting at 51% acetonitrile, whereas the chymotrypsin reaction contained a closely spaced doublet eluting at the same position. Analysis by Edman degradation and m.s. indicated that the trypsin-generated peak consisted of the peptide from Met³⁷⁷ to the C-terminus of α_2 -AP, whereas the chymotrypsin-generated peak contained two peptides, one from Ser³⁷⁸ to the C-terminus and the other from Ser³⁷⁵ to the C-terminus (Figure 4). Thus reaction between trypsin and α_2 -AP resulted in cleavage of Arg³⁷⁶-Met³⁷⁷, whereas

Figure 3 Titration of α_2 -AP with trypsin (a and c) and chymotrypsin (b and d) visualized by SDS/PAGE (top panels) and non-denaturing PAGE (bottom panels)

α_2 -AP samples were incubated with trypsin or chymotrypsin for 5 min at 23 °C in assay buffer, followed by addition of DCl or chymostatin to inhibit excess trypsin or chymotrypsin respectively. In each gel, lane 1 is untreated α_2 -AP; lanes 2–7 are 0.2–1.2 molar equivalents of enzyme on a weight basis. Lane 8 is a control with α_2 -AP treated under the same conditions as lane 7, except that the enzymes had been inactivated by DCl or chymostatin before addition of α_2 -AP. Lanes 9 in (c) and (d) show the migration of α_2 -AP that had been inactivated via cleavage of the RSL by a 0.01 molar equivalent of neutrophil elastase. Lanes 10 of (a) and (b) show the migration of DCl-treated trypsin and chymotrypsin respectively, the same amount as was added to lanes 7 and 8.

reaction between chymotrypsin and α_2 -AP resulted in cleavage of Met³⁷⁴-Ser³⁷⁵ (site 1) and Met³⁷⁷-Ser³⁷⁸ (site 2). This confirms the work of Potempa et al. (1988), who showed that α_2 -AP has overlapping reactive sites for the two proteinases, although these authors did not report the site 1 cleavage. Though trypsin only caused one cleavage in the RSL, chymotrypsin caused two (Figure 4). The ratio of the two chymotrypsin cleavages varied with temperature, as did the chymotrypsin-inhibitory capacity of α_2 -AP (Table 1).

We conclude that reaction of chymotrypsin with α_2 -AP results in cleavage at two sites in the RSL, visualized when reaction mixtures are rapidly denatured in TFA, each one distinct from the trypsin cleavage site (Figure 4). Quantification of the two chymotryptic RSL cleavages revealed that more hydrolysis at site 1 occurred at 37 °C, concomitant with decreased inhibitory capacity (see Table 1).

Are there distinct trypsin- and chymotrypsin-inhibitor populations of α_2 -AP?

Since proteinase inhibitors do not usually have overlapping reactive sites, we considered the possibility that the α_2 -AP preparations contained two distinct populations with separate specificities. This was done by saturating α_2 -AP with trypsin, followed by titration with chymotrypsin (Figure 5). We observed a small amount of chymotrypsin inhibitory activity in trypsin excess. We do not know the reason for this, but it is inconsistent with separate populations since if this were the case, the chymotrypsin titration should have been linear and not dependent upon trypsin reaction with α_2 -AP. We cannot say that two populations do not exist, but if they do, they must equilibrate in less than one minute: the time taken to complete the trypsin titration in this essay.

Figure 4 Diagram of the cleavage sites in α_2 -AP caused by trypsin and chymotrypsin

Purified human plasma α_2 -AP is processed by an unknown mechanism at the Pro¹²-Asn¹³ bond so that 50–67% of molecules lack the N-terminal dodecapeptide. Chymotrypsin cleaves at two sites near the N-terminus, and at two sites in the RSL. The N-terminal cleavages are thought to be fortuitous, but cleavage at site 1 in the RSL inactivates the inhibitor. Site 2 in the RSL is the inhibitory site. The trypsin inhibitory site is adjacent to the chymotrypsin site, so close that binding of the two proteinases cannot take place simultaneously.

Table 1 Disproportionation of chymotrypsin inhibition by α_2 -AP at elevated temperature

In the site 1:site 2 cleavage data the relative amount of each peptide was calculated from the gross recovery of Pth-Leu (site 1 cleavage) and Pth-Phe (site 2 cleavage) in cycle five of the Edman degradation. The ratio is calculated from the average of four sequence runs per sample, and relates to the disproportionate cleavage of site 1 at 37 °C. The inhibitory ratio of α_2 -AP/chymotrypsin is calculated from results in Figure 2, and relates to the amount of α_2 -AP required to inhibit 1 mol of chymotrypsin. Abbreviation: Pth, phenylthiohydantoin.

Temperature ...	0 °C	37 °C
Ratio of site 1:site 2 cleavage	0.68	0.93
Ratio of α_2 -AP:chymotrypsin	0.9	1.6

Figure 5 Sequential titration by trypsin and chymotrypsin was used to determine whether two independent inhibitory species of α_2 -AP exist

A fixed concentration of trypsin was allowed to react with various increasing concentrations of α_2 -AP for 1 min at 23 °C, a fixed concentration of chymotrypsin was added, followed 1 min later by either the trypsin or chymotrypsin substrate. Residual trypsin (●) or chymotrypsin (○) activity was plotted against the molar concentration of α_2 -AP relative to trypsin.

DISCUSSION

Though the N-terminus of virgin α_2 -AP has been reported to be an Asn residue (Wiman and Collen, 1979; Potempa et al., 1988), about one third to one half of the inhibitor isolated in our laboratory contained Met as the N-terminal residue, implying a different signal peptidase cleavage site from that previously suggested by Holmes et al. (1987). Since the Ala-Met bond is preceded by a better signal peptidase recognition site (Kozak, 1984) than the Pro-Asn bond (see Figure 1), it is likely that the N-terminus seen by previous authors is a result of proteolytic processing that occurs during transport or during circulation of the protein. Sumi et al. (1989) noticed that recombinant α_2 -AP expressed in hamster kidney cells contained the N-terminal-extended sequence, and suggested that this represents a propeptide of the inhibitor. Our demonstration of the existence of this sequence in normal plasma α_2 -AP argues against this, though it is interesting that the full-length form cross-links less effectively to fibrin (Sumi et al., 1989).

Determination of the target proteinase-induced site of peptide-bond cleavage in the RSL of serpins is usually used to diagnose the P₁ site of the inhibitor. It is not known when cleavage of the bonds takes place but, by analogy with other serpins, it is likely that peptide-bond scission is a result of denaturing conditions, and that the bond is intact in the native complex (Travis and Salvesen, 1983; Longstaff and Gaffney, 1991; Mast et al., 1991b; Matheson et al., 1991; Bode and Huber, 1992). Chymotrypsin caused two cleavages in the α_2 -AP RSL, at site 1 and at site 2. The site 1 cleavage was not reported by Potempa et al. (1988), who may have missed it since it is only transiently detectable, and since their incubations were much longer than ours. In our hands, longer incubations lead to removal of the Ser³⁷⁵-Met³⁷⁷ tripeptide even in the presence of excess α_2 -AP, presumably following dissociation of chymotrypsin from its relatively loose complex with the inhibitor. Since chymotrypsin interacted with two sites in the RSL, we considered which one caused inhibition.

Cleavage in the RSL outside the precise reactive centre leads to inactivation of serpins (Mast et al., 1992). In the case of α_2 -AP, cleavage at site 1 was more apparent at 37 °C than at 0 °C (Table 1), and this correlated with a decrease in inhibitory capacity at 37 °C. It is therefore unlikely that site 1 cleavage represents another inhibitory site for chymotrypsin; rather, cleavage simply causes inactivation of the inhibitor. It is more likely that the two observed cleavages in the RSL represent competition between two sites on α_2 -AP for reaction with chymotrypsin (Figure 4). Such a competition has been reported during the reaction of human chymase with α_1 -antichymotrypsin and α_1 -PI (Schechter et al., 1989) in which cleavage could occur several residues upstream or downstream of the reactive centre. The competition was influenced by pH and ionic strength. Patston et al. (1991) noticed that reaction of human C1-inhibitor with human plasma kallikrein resulted in two pathways, one of which led to inactivation by cleavage in the RSL and one of which led to inhibition. These reactions were dependent on temperature of incubation such that C1-inhibitor became a better inhibitor as the temperature increased from 0 °C to 37 °C, contrary to our observation with α_2 -AP. On the basis of this observation the authors concluded that serpins are suicide substrates (mechanism-based inactivators).

It is difficult to determine the mechanism of serpins from results generated by observing a single interaction, since our results gave an opposite temperature effect to those of Patston et al. (1991). Moreover, we believe the mechanism of action of serpins can be explained by a description that takes into account

the overlapping reactive centres of α_2 -AP (which deviates from standard-mechanism inhibitors), and the temperature and pH effects of inhibition versus inactivation of α_1 -PI, α_1 -antichymotrypsin, α_2 -AP and C1-inhibitor. The standard mechanism of inhibition of serine proteinases by proteins such as those of the kunin and kazal families can be envisioned as close to a lock and key interaction between the RSLs of the inhibitors and the substrate-binding sites of the enzymes. In most cases the RSL main chain conformations in the P_2 - P_2' regions are superimposable and offer rigid structures complementary to the enzyme-binding sites, so that many unrelated proteins seem to have evolved closely similar binding loops (Read and James, 1986; Bode and Huber, 1992). However, recent solution structures of the proteinase inhibitor eglin C, not related to serpins, demonstrated a mobile RSL (Hyberts et al., 1992), much more so than had previously been described by X-ray crystallography. The mobility of eglin C is sufficient for its RSL to adopt substantially different conformations in solution, though in its complex with proteinases the RSL superimposes well with kunin and kazal RSLs. This leads us to modify our view of the flexibility of RSLs.

It has been suggested before that the RSLs of serpins may be more mobile than the apparently rigid loops of the standard-mechanism inhibitors (Carrell et al., 1991; Mast et al., 1992). The recent results on the mobility of the RSL of eglin C give a precedent for this, and we suggest that the RSLs of serpins also are mobile, and that the reactive centre main chains sample space in solution. In the special case of α_2 -AP, some conformations of the loop favour trypsin binding, others favour chymotrypsin binding. Alterations in the environment of the RSL change the rate of sampling to favour certain conformations. Some of these conformations facilitate an inhibitory function, others allow proteolysis leading to inactivation, so that a conformation that allows reaction of chymotrypsin with site 2 in α_2 -AP is more populated at 0 °C than at 37 °C. When a proteinase binds to an inhibitory conformation, the RSL probably becomes locked in the canonical conformation for RSLs typically seen in X-ray structures of proteinase/inhibitor complexes, as described by Bode and Huber (1992). This mechanism requires no peptide-bond cleavage of this serpin in its inhibitory complex with a proteinase.

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