Interaction of subtilisins with serpins

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Abstract

Serpins are well-characterized inhibitors of the chymotrypsin family serine proteinases. We have investigated the interaction of two serpins with members of the subtilisin family, proteinases that possess a similar catalytic mechanism to the chymotrypsins, but a totally different scaffold. We demonstrate that α_1 proteinase inhibitor inhibits subtilisin *Carlsberg* and proteinase K, and α_1 antichymotrypsin inhibits proteinase K, but not subtilisin *Carlsberg*. When inhibition occurs, the rate of formation and stability of the complexes are similar to those formed between serpins and chymotrypsin family members. However, inhibition of subtilisins is characterized by large partition ratios where more than four molecules of each serpin are required to inhibit one subtilisin molecule. The partition ratio is caused by the serpins acting as substrates or inhibitors. The ratio decreases as temperature is elevated in the range 0–45 °C, indicating that the serpins are more efficient inhibitors at high temperature. These aspects of the subtilisin interaction are all observed during inhibition of chymotrypsin family members by serpins, indicating that serpins accomplish inhibition of these two distinct proteinase families by the same mechanism.

Keywords: inhibitory mechanism; partition ratio; proteinase inhibitor; serpin; subtilisin

The subtilisin and chymotrypsin families are examples of convergent evolution where distinct protein scaffolds are used to position essentially identical catalytic machineries (Dayhoff et al., 1978; Rawlings & Barrett, 1994). Substrates bind in an extended conformation to a cleft on the surface of the proteinases with their scissile bond positioned close to the catalytic serine. Because solved structures of substrates bound to proteinases are extremely rare, the structural determinants of substrate binding are best appreciated when the features of inhibitor/proteinase complexes are compared.

Subtilisin *Carlsberg* and α -chymotrypsin are representative members of each of the families, and both have been crystallized in complex with a number of protein inhibitors, for example eglin c. The reactive site loop (RSL) of eglin c adopts a very similar conformation in each of the two complexes (Read & James, 1986; Bode & Huber, 1992). Moreover, when the cata-

lytic residues of SCARL and chymotrypsin are optimally superimposed, the RSLs occupy close to identical space. This signifies that eglin c inhibits members of the two proteinase families by the same mechanism. It is supported by solution kinetic data showing that members of both families are inhibited, often with very similar binding constants, by the same protein inhibitors (Read & James, 1986; Bigler et al., 1993; Greagg et al., 1994). Therefore, the subtilisins and chymotrypsins not only have a similar mechanism of catalysis, they also have a similar mechanism of binding to inhibitors, such as eglin c, that obey the standard mechanism of inhibition (Laskowski & Kato, 1980).

When comparing subtilisins with chymotrypsins, there has been a general focus on standard mechanism protein inhibitors that include members of the Kazal, eglin, and *Streptomyces* subtilisin inhibitor families (Read & James, 1986; Takeuchi et al., 1991; Bode & Huber, 1992; Bigler et al., 1993). There has been very little focus on the serpins, which may not operate by the standard mechanism and which have been studied almost exclusively with the chymotrypsin family. Indeed, we could only find one report of the interaction of subtilisins with serpins (Abe & Kuromizu, 1989). This study concluded that subtilisin BPN' (75% sequence identity with SCARL) was inhibited by the serpin α_1 proteinase inhibitor, but that the mechanism differed from chymotrypsin inhibition by the same serpin. In addition to this study, there have been reports of the inhibition of pro-protein processing proteinases by natural and synthetic variants of α_1 PI (Anderson et al., 1993; Brennan & Nakayama, 1994;

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Abbreviations: α_1 PI, α_1 proteinase inhibitor; α_1 ACT, α_1 antichymotrypsin; cat G, cathepsin G; DTT, dithiothreitol; HNE, human neutrophil elastase; pNA, p-nitroanilide; PROK, *Thermus albus* proteinase K; RSL, reactive site loop; SCARL, *Bacillus licheniformis* subtilisin *Carlsberg*; SSI, *Streptomyces* subtilisin inhibitor; TCA, trichloroacetic acid; TUG, transverse urea gradient.

Watanabe et al., 1995). These proteinases are relatives of the subtilisins, and are selective for substrates containing paired basic residues (Barr, 1991; Siezen et al., 1991).

The serpin fold contains 9 α -helices and 3 β -sheets, describing a scaffold that is significantly larger and more complex than those of the standard mechanism inhibitors (Loebermann et al., 1984; Stein & Carrell, 1995). The latter have a rather rigid RSL, which the binding cleft of target proteinases accommodates without any major conformational rearrangements (Read & James, 1986; Bode & Huber, 1992). In contrast, recent studies of the structure and mechanism of serpins suggest that the serpin RSL is in a much more flexible conformation (reviewed by Stein & Carrell, 1995). Thus, considerable adaptation to the proteinase's substrate cleft may occur during complex formation—setting the serpins apart from the other serine proteinase inhibitor families. Structural and kinetic data have led some workers to propose very different inhibitory mechanisms that often include a requirement for peptide bond scission in serpins, at the P₁-P₁' bond, not seen in the standard mechanism inhibitors (reviewed by Wright & Scarsdale, 1995).

To explore the serpin inhibitory mechanism, we have determined whether they inhibit subtilisins. Are the serpins like the standard mechanism inhibitors in their ability to inhibit members of both serine proteinase families, or does the distinct subtilisin scaffold preclude inhibition? We have used two distantly related subtilisins: SCARL from a prokaryote and proteinase K from a eukaryote. We report the characteristics of the interaction of these subtilisins with two human serpins, α_1 PI and α_1 ACT, which have been characterized extensively as inhibitors of the chymotrypsin family proteinases.

Results

Inhibition

The ability of α_1 PI and α_1 ACT to inhibit the subtilisins was checked initially by titrating the enzymes with the serpins. Following incubation at 37 °C for 15 min to allow complex formation, residual proteinase activity was determined as described in the Materials and methods and the results are presented in Figure 1. Both α_1 PI and α_1 ACT inhibited PROK, but SCARL was only inhibited by α_1 PI under the indicated conditions. The decrease in activity of SCARL by α_1 ACT cannot be ascribed definitively to inhibition because very high protein concentrations are required (see Fig. 1B, inset). It may simply be due to the serpin acting as a competing substrate in the reaction.

Stoichiometry of inhibition

The active concentration of individual proteinases was determined with eglin c that had been standardized with active-site titrated chymotrypsin. The concentration of α_1 PI was determined with standardized human neutrophil elastase (HNE, a chymotrypsin family member) and that of α_1 ACT with chymotrypsin and cathepsin G (cat G, a chymotrypsin family member) under conditions that are known to result in equimolar complex formation with no turnover of the inhibitors (Salvesen & Nagase, 1989; Patston, 1995). Comparison of the protein concentration calculated from absorbance at 280 nm and inhibitory concentration showed the serpins to be 85% active by weight. As seen in Figure 1, the amount of serpin required to inhibit the

Fig. 1. Titration of SCARL and PROK by α_1 PI and α_1 ACT. **A:** Subtilisins (1.5×10^{-7} M) were incubated with a range of α_1 PI concentrations in a total volume of 100 μ L for 15 min at 37 °C. Residual proteinase activity was determined by spectrophotometric analysis using the substrate suc-Gly-Gly-Phe-pNa as described in the Materials and methods. **B:** A similar analysis is shown for α_1 ACT. The inset was derived from an experiment containing 2.8×10^{-10} M SCARL. This low enzyme concentration was required to allow maximization of the I/E ratio. Abscissae are described in terms of the inhibitor/enzyme ratio using values of active serpins determined by titration with HNE or chymotrypsin, and subtilisins by titration with standardized eglin c.

subtilisins is much higher than that required to inhibit HNE or cat G. It appears that several molecules of each serpin are consumed to inhibit one molecule of the proteinase. The ratio of the inhibitor to the proteinase at the point of zero residual activity is defined as the partition ratio (Patston et al., 1991).

Individual partition ratios are described by the intersection of the titration lines with the abscissa in Figure 1. To avoid possible errors in the activity assay due to proteolytic impurities in commercial preparations of subtilisins, we purified subtilisins from different commercial sources as described in the Materials and methods. SCARL and PROK purified from different sources gave identical partition ratios with α_1 PI and α_1 ACT within experimental errors, suggesting that the partition ratios

obtained here were not due to contaminating proteinases in our samples.

The stability of the inhibited complexes was determined by incubating proteinase with inhibitor at a ratio that gave 50% residual enzyme activity. The residual activity did not change during 8 h incubation at 37 °C (not shown), indicating that the serpins are not temporary inhibitors and that the inhibited complex is too stable to enable determination of equilibrium constants.

SDS-PAGE analysis

The interactions of the serpins with the subtilisins were visualized by PAGE analysis. A set amount of each serpin was allowed to incubate with varied amounts of proteinase at 37 °C for 15 min. The amounts of serpin required to inhibit one mol equivalent of proteinase was determined by titration as described in Figure 1. This value was used to calculate the enzyme/inhibitor ratios described in Figure 2 and subsequent figures. Samples that contained subtilisins and serpins added to SDS sample buffer and then boiled exhibited variable amounts of complex formation when analyzed by SDS-PAGE. To observe reproducible results, we found it essential to terminate proteolytic activity rapidly before addition of SDS. This was accomplished by trichloroacetic acid treatment as described in the Materials and methods. As the amount of proteinase is increased, we observe

proteolysis in parallel with complex formation. For example, the 52-kDa α_1 PI band splits to a 47-kDa derivative and an 80-kDa derivative as the concentration of SCARL is increased (Fig. 2A). The 47-kDa derivative is consistent with cleavage within the serpin's RSL, and the 80-kDa derivative is consistent with a proteinase/serpin complex. The intensity of this band probably represents the relatively small amount of complex formed during a reaction in which much of the serpin acts as a substrate for the proteinase. This point is expanded in the Discussion. To confirm the identity of the 80-kDa band, we labeled SCARL with 125 I, incubated it with α_1 PI, and visualized the result by phosphor imaging (Fig. 2E). Quantitative analysis revealed that 95% of the entire radio label in lane 1 was contained in the 80-kDa band. Therefore, in inhibitor excess essentially all SCARL forms a stable complex with α_1 PI that resists 10% TCA treatment, and boiling for 5 min in the presence of 1% SDS and 50 mM DTT.

An equivalent pattern is seen when α_1 PI is incubated with PROK (Fig. 2B), indicating similar complex formation and proteolysis. In the case of the PROK interaction with α_1 ACT (Fig. 2C), we observe the appearance of a 97-kDa band, presumably representing the PROK/ α_1 ACT complex. As noted previously, α_1 ACT that has been cleaved in the RSL does not change its rate of migration in our SDS-PAGE system (Mast et al., 1991). Consequently, cleavage was monitored by non-

Fig. 2. PAGE analysis of the interaction of subtilisins with α_1 PI and α_1 ACT. In each experiment, a set amount of serpin was incubated with a range of proteinase concentrations for 15 min at 37 °C. Reactions were terminated by addition of phenylmethane sulfonyl fluoride to a concentration of 2 mM. Samples for SDS-PAGE were first treated with TCA as described in the Materials and methods. Gels were stained with Coomassie blue R250 to visualize the resulting protein components. The migration position of protein standards is shown on the left, and the subtilisin is shown in the right hand lanes. Figures between gels identify the proteinase/inhibitor molar ratio based on the amount of inhibitor required to inhibit one mol equivalent of the subtilisin, i.e., compensating for the partition ratio. **A:** α_1 PI (8.8 μ M) titrated with SCARL followed by SDS-PAGE; **B:** α_1 PI (8.8 μ M) titrated with PROK followed by SDS-PAGE. α_1 ACT (6.7 μ M) was titrated with PROK and samples were divided into equal portions for analysis by SDS-PAGE (**C**) and non-denaturing PAGE (**D**). **E:** 125 I-labeled SCARL incubated with a 10-fold molar excess of α_1 PI (final concentration 5.3 μ M, lane 1), run in SDS-PAGE followed by autoradiography to detect migration of the proteinase complex. Lane 2, SCARL was treated with phenylmethylsulfonyl fluoride (2 mM, 15 min) before addition of α_1 PI; lane 3, no α_1 PI added.

denaturing PAGE of the reaction products before TCA treatment (Fig. 2D). We observed no difference in the gel profiles in the presence or absence of 50 mM DTT for any of the interactions (not shown).

TUG-PAGE analysis

We analyzed the products of the interaction of SCARL with α_1 PI by TUG-PAGE, a technique that is used to describe the conformational stability of proteins (Fig. 3A). Three species were seen, one corresponding to the unfolding signature typical of virgin α_1 PI, and two that did not unfold. The lower band is attributable to RSL-cleaved α_1 PI, and the upper one probably represents the SCARL/ α_1 PI complex. This was confirmed when 125 I-labeled SCARL was used to allow complex formation (Fig. 3B). The SCARL/ α_1 PI complex does not unfold and has the conformational stability typical of complexes between serpins and chymotrypsin family members (Mast et al., 1991).

Sequence determination of the cleavage sites

Evidence from the PAGE analyses implied that a large portion of α_1 PI was cleaved by SCARL during the interaction. The location of the cleavage site was determined by N-terminal sequence analysis of components separated by PAGE. Efficient separation of the components from unreacted α_1 PI was difficult to achieve using nondenaturing or SDS-PAGE, so we took advantage of the TUG-PAGE profile shown in Figure 3A. There we see that clear separation is achieved at urea concentrations above 4 M. We cast gels of 7% acrylamide containing 5 M urea to separate the cleaved and uncleaved α_1 PI derivatives. α_1 PI (10^{-5} M) was incubated with SCARL (5×10^{-7} M) at 37 °C for 5 min, and the reaction was stopped by adding SSI (10^{-5} M). This reaction condition was designed to minimize excess proteolysis and to allow determination of the initial cleavage of α_1 PI. The reaction mixtures were divided into two parts; one for PAGE in 5 M urea (Fig. 4A, bottom), and another for SDS-PAGE (Fig. 4A, top). A similar analysis was performed to resolve products of the incubation of α_1 PI with PROK (Fig. 4B). Minimal complex formation and cleavage was observed with the

E/I ratios 0.33 for SCARL and 0.75 for PROK. These reaction conditions were scaled up sixfold. Protein bands were stained briefly by Coomassie blue following blotting to polyvinylidene difluoride membrane (Matsudaira, 1987). The 10 N-terminal residues of the complex bands from SDS-PAGE, or the cleaved bands from 5 M urea PAGE were determined by using an Applied Biosystems 476A Protein Sequencer. For both SCARL and PROK, the sequence data allowed assignment of two cleavage sites: 44% of the yield was accounted for by cleavage at Met 358–Ser 359, and 56% was accounted for by cleavage at Met 351–Phe 352. These data indicate that partitioning of α_1 PI results from cleavage at one or two positions in the RSL. When the reaction of SCARL with α_1 PI was allowed to proceed longer at higher enzyme concentration, an additional cleavage was detected between residue Thr 11 and Asp 12 in the N-terminal region of α_1 PI.

The reaction conditions contained inhibitor in large excess, and a short incubation time to minimize the possibility of adventitious cleavages. Although we have taken care to observe initial cleavages, we cannot rule out that partitioning results from initial cleavage at Met 351, followed by an adventitious cleavage at Met 358. We can rule out initial cleavage at Met 358 followed by cleavage at Met 351 because sequence data from the Met 351 site revealed good yields for residues 359–361. Significantly, the relative yield of the two SCARL cleavage sites in the RSL of α_1 PI was the same at 0 °C and 37 °C.

Sequence analysis of the SCARL/ α_1 PI complex band in SDS-PAGE (Fig. 4A) revealed equal yields of the N-terminal sequences of the proteinase and α_1 PI. Analysis of this complex from nondenaturing PAGE (Fig. 4C) revealed three sequences: the N-termini of α_1 PI and SCARL and an additional sequence corresponding to cleavage at Met 358. Cleavage at Met 358 results in formation of the C-terminal fragment of about 4 kDa that remains an integral part of the complex, but is dissociated by SDS treatment. The locations of the cleavage sites are illustrated schematically in Figure 5. When the proteinases were pre-inactivated with phenylmethane sulfonylfluoride, no complex formation and no proteolysis was seen (Fig. 4), indicating that only the active proteinases contribute to proteolysis and complex formation.

Fig. 3. TUG-PAGE analysis of α_1 PI interaction with 125 I-SCARL. SCARL that had been labeled with 125 I was incubated with an approximately eightfold molar excess of active α_1 PI (1.2×10^{-5} M) for 15 min at 37 °C. The reaction was terminated with phenylmethane sulfonylfluoride (2 mM). The sample was applied to a 7% acrylamide gel containing a 0–8 M transverse urea gradient (see the Materials and methods). The gel was stained, dried, and phosphorimaged as described in the Materials and methods. **A:** Stained gel showing the location of all protein components. **B:** Phosphorimage showing location of radiolabeled SCARL in complex with α_1 PI.

Fig. 4. Separation of subtilisin-generated α_1 PI products for sequence analysis. α_1 PI (10^{-5} M) was incubated with (A) SCARL or (B) PROK for 5 min at 37°C , followed by addition of SSI to a concentration of 10^{-5} M to terminate the reaction. Samples were divided and run immediately in 5 M urea PAGE (bottom panels) or treated with TCA for electrophoresis in SDS-PAGE (top panels). Lanes marked by asterisks indicate products from reactions when the proteinase was preincubated with phenylmethylsulfonyl fluoride before addition of α_1 PI. C: A portion of the SCARL/ α_1 PI reaction mixture prepared under condition that gave an E/I ratio of 0.33 was run in nondenaturing PAGE. All gels were stained with Coomassie blue to visualize proteins.

Temperature dependence of the partition ratio

We investigated the temperature dependence, in the range 0 – 55°C , of the partition ratio of α_1 PI and α_1 ACT with SCARL

Fig. 5. Cleavage sites in α_1 PI. The drawing depicts α_1 PI, not to scale, showing the location of the RSL (defined here as the region Ala 348–Glu 363) in black. This is the region that limited proteolysis studies have demonstrated to be exposed in the native, inhibitory conformation (Mast et al., 1992). SCARL and PROK both cleave at the sites marked by arrows and the associated numbers indicate the fraction of cleavage at each site. The fraction is the same for both proteinases, and is independent of temperature in the range 0 – 37°C for SCARL (see text). Sequence analysis of the SCARL/ α_1 PI complex indicates cleavage at the Met 358–Ser 359 bond, which is the same location as cleavage observed in complexes of α_1 PI with chymotrypsins (Travis & Salvesen, 1983). Thus, Met 358 defines the P_1 residue for complex formation with SCARL and chymotrypsins. The secondary cleavage site (see text) is designated by the open arrow.

and PROK (Fig. 6). Serpins and subtilisins were allowed to reach the desired temperature and then titrated at that temperature as described in the Materials and methods. Residual proteinase activity was determined at 37°C as described in the Materials and methods. The subtilisins are stable in the range of 0 – 55°C during the incubation time of this experiment. Therefore, the temperature dependence of the partition ratio reflects the temperature dependence of the inhibitory activity of α_1 PI and α_1 ACT. Beyond 45°C , α_1 PI starts to polymerize as visualized previously on native-PAGE (Mast et al., 1992), and the inhibitory activity drops.

Because the proportion of cleavages by SCARL in α_1 PI at Met 351 and Met 358 was the same at 0°C and 37°C , the decrease in the partition ratio must reflect an increase in the number of conformations favorable for inhibition.

Determination of the association rate constant (k_{ass}) at 37°C

We determined the association rate (k_{ass}) for serpin–subtilisin interactions at 37°C , correcting for the partition ratio at this temperature. The observed rate of inhibition (k_{obs}) was deter-

Fig. 6. Temperature dependence of the partition ratio. Partition ratios were determined as described in the legend to Figure 1. Components were pre-incubated at the required temperature for at least 5 min before analysis. (●) α_1 PI/SCARL, (○) α_1 PI/PROK, (+) α_1 ACT/PROK. The ordinate was extended discontinuously at low temperature to illustrate the high partition ratio for α_1 ACT/PROK.

mined from progress curve analysis (Knight, 1986) as described in the Materials and methods (see Fig. 7 for representative data). This was used to determine the second-order rate of inhibition (k_{ass}) according to the relationship described in the Materials and methods, having calculated K_m values for each proteinase on the appropriate substrate at 37 °C. Values of k_{ass} were $1.2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ for α_1 PI-SCARL, $1.4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ for α_1 PI-PROK, and $1.1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ for α_1 ACT-PROK.

Discussion

Inhibitor binding by subtilisins and chymotrypsins has been studied most extensively at the structural level by using the inhibitor eglin c (Read & James, 1986; Bode & Huber, 1992), and at the kinetic level by using avian ovomucoid third domains (Laskowski et al., 1987; Bigler et al., 1993). It is clear from both types of study that the binding is equivalent, but certain distinctions are evident. These distinctions in inhibitor binding illustrate the characteristic differences in substrate hydrolysis by members of the two families

In each case, a short stretch of the peptide backbone of the inhibitor forms an antiparallel β -sheet with main-chain strands on the proteinase's surface. Pockets on the proteinase surface secure the correct alignment by binding side chains of the substrate or inhibitor. Although they use these similar binding modes, subtilisins and chymotrypsins are distinguished by the following: First, two subtilisin strands participate in β -sheet formation with the inhibitor's main chain, whereas chymotrypsins accomplish substrate binding with the use of a single strand (re-

Fig. 7. Example of a progress curve analysis. Progress curve analysis of inhibition was measured as described in the Materials and methods. PROK ($3.5 \times 10^{-11} \text{ M}$) was incubated at in the presence of suc-Ala-Ala-Pro-Phe-pNa 37 °C (1) without, or with the following total protein concentrations of α_1 PI: (2) 1.4 nM, (3) 2.1 nM, (4) 2.8 nM, (5) 3.5 nM. **A:** Product release measured by absorbance at 405 nm. The final product concentration (P_∞) was taken as the plateau value in curves 3, 4, and 5. Curve 2 was not further analyzed because inhibition was not complete by the end of the analysis. **B:** Curves 3, 4, and 5 were transformed to give k_{obs} by plotting $\ln(P_\infty - P_t)$ against time as described in the Materials and methods.

viewed by Perona & Craik, 1995). Second, binding energy and specificity are dominated by S_1 in chymotrypsins (Perona & Craik, 1995), but in subtilisins S_1 and S_4 interactions contribute almost equally (Moriyama et al., 1970; Robertus et al., 1972; Grøn & Breddam, 1992; Grøn et al., 1992).

We chose to examine SCARL and PROK because they are the most distantly related members of the subtilisin family whose structures are known (44% sequence identity, 0.888 Å RMS deviation of superimposed $C\alpha$ -atoms [Siezen et al., 1991]). Thus, there is a good chance that the interactions these two subtilisins

share will hold for the whole family. We show that α_1 PI inhibits both SCARL and PROK, but α_1 ACT only inhibits PROK. As observed with the chymotrypsins, inhibition is too tight to enable determination of equilibrium constants. However, under pseudo-first-order reaction conditions (Salvesen & Nagase, 1989), inhibition was consistent with second-order kinetics. The rates of inhibition (k_{ass}) are in the $10^5 \text{ M}^{-1} \text{ s}^{-1}$ range, intermediate to those observed with chymotrypsin and α_1 PI, $6 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, or α_1 ACT, $6 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ (Beatty et al., 1980).

PAGE analyses show that the portion of α_1 PI and α_1 ACT that inhibits subtilisins does so by forming complexes with the proteinases that resist boiling in the presence of DTT and SDS (Fig. 2). This is typical of the interaction of serpins with chymotrypsins (Travis & Salvesen, 1983), and implies that inhibition results in the same stable complex for both serine proteinase families.

Our conclusion contrasts to the interpretation of Abe and Kuromizu (1989), who investigated the interaction of α_1 PI with subtilisin BPN'. These authors concluded, based primarily on SDS-PAGE analysis, that the subtilisin BPN'/ α_1 PI complex was not as stable as the chymotrypsin/ α_1 PI and that it did not result in covalent bond formation. We analyzed the stability of the subtilisin BPN'/ α_1 PI complex and found that it indeed did resist SDS-PAGE in the presence of DTT (not shown), as demonstrated for the other two subtilisins. Subtilisins are very resistant to denaturation, and their catalytic activity must be terminated before SDS-PAGE to avoid adventitious proteolytic activity during denaturation (see the Results and the Materials and methods). Most likely, Abe and Kuromizu did not detect SDS- and DTT-resistant complexes due to proteolysis by excess enzyme during the preparation of their samples.

Upon complex formation with SCARL, α_1 PI adopts a conformation that resists unfolding in TUG-PAGE (Fig. 3). This stabilization following complex formation is typical of complexes between chymotrypsin family members and serpins (Mast et al., 1991). Therefore, we conclude that serpins inhibit subtilisins to give complexes with the kinetic and conformational stability characteristic of serpin/chymotrypsin family interactions. This implies an identical mechanism of inhibition. However, with both subtilisins, the serpins act as substrates as well as inhibitors, and this is reflected by the partition ratios.

Of the active site-directed protein inhibitors, only serpins exhibit partitioning, which is a reflection of the flexibility of the RSL. When the effect of proteinases on the serpins is examined, one observes two extremes. Proteinases that are not inhibited cleave the RSL; the newly generated terminals adopt positions in the opposite ends of the molecule and are unable to reassociate. An example of this is the action of HNE on α_1 ACT (Morii & Travis, 1983). In the other extreme, the reacting proteinase is inhibited and does not progress through the substrate pathway, for example, the reaction of HNE with α_1 PI (Travis & Salvesen, 1983). Some proteinases are intermediate and the place they adopt between the extremes defines the partition ratio.

Partitioning between the inhibitor and substrate pathways is seen for the interaction of serpins with some chymotrypsin family members (Patston et al., 1991; Gettins et al., 1992; Cooperman et al., 1993; Enghild et al., 1993; Schechter et al., 1993). However, it is not usually as extreme as shown here with the subtilisins unless specific mutations are made in the serpin that cause it to favor the substrate pathway (see Hood et al., 1994, for example). The chymotrypsin family member most similar to sub-

tilisins in terms of partitioning is human mast cell chymase, which has partition ratios of 5 for α_1 PI and 4.5 for α_1 ACT at 25 °C (Schechter et al., 1989).

Partitioning is most frequently due to cleavage predominating over inhibition at the P₁ site (Patston et al., 1991; Cooperman et al., 1993). Occasionally, cleavage outside the P₁ inhibitory site, but within the RSL, causes partitioning (Schechter et al., 1989; Enghild et al., 1993). For the interaction of subtilisins with α_1 PI, both appear to occur and there are two components of the partitioning. One is due to cleavage of the RSL at Met 351. The other component is due to cleavage at Met 358. Cleavage at either site results from binding of Ala in S₄ and Met in S₁ (see Fig. 5), thus both sites satisfy the subtilisin substrate preference equally. Inhibition of SCARL by α_1 PI results from binding of Met 358 to the enzyme's S₁ pocket, thus cleavage and inhibition occur at the same site.

Partitioning is due to cleavage predominating over inhibition. Some authors have concluded that this may represent the results of serpins acting as mechanism-based inactivators (Patston et al., 1991; Gettins et al., 1992; Lawrence et al., 1995). In this case, Met 358-Ser 359 cleavage would be an absolute requirement for inhibition and the inhibited proteinase would exist with its catalytic serine acylated by the α -carbonyl of Met 358 (Lawrence et al., 1995; Wright & Scarsdale, 1995). Others have concluded that cleavage may be a result of the denaturing conditions required to visualize it and that the observed acylation (SDS-stable complex formation) is an artifact and equivalent to a trapped intermediate in the substrate hydrolysis pathway (Travis & Salvesen, 1983; Longstaff & Gaffney, 1991; Mast et al., 1991; Stein & Carrell, 1995). In this case, the serpins would be more like the standard mechanism inhibitors.

As temperature increases, the partition ratio declines, indicating more faithful inhibition. Recent X-ray structures of intact antithrombin (Carrell et al., 1994; Schreuder et al., 1994) and α_1 ACT (Wei et al., 1994) reveal frozen noninhibitory conformations that would require significant movement to adapt the RSL efficiently to the substrate sites of a target proteinase before inhibition can occur. The decline of the partition ratio as temperature increases is due to the inhibitory pathway becoming more dominant. Therefore, the simplest explanation for the decline in the partition ratio is a shift in conformations of the RSL from substrate-like to inhibitory at the elevated temperatures. Such shifts would probably be restricted to the local area of the RSL because global conformational changes in the serpin C1-inhibitor are not seen in the range 4–38 °C (Patston et al., 1991).

We show that serpins inhibit subtilisins. This may explain the inhibition of some of the proprotein convertases that include mammalian homologues of the microbial subtilisins (Anderson et al., 1993; Brennan & Nakayama, 1994). These convertases have diverged considerably from the subtilisins and possess specificity for basic residues in S₁, S₂, and S₄. Significantly, they are not inhibited by wild-type α_1 PI, but by a natural variant of α_1 PI that contains a Met 358 Arg substitution (Brennan & Nakayama, 1994), or by synthetic α_1 PI variants containing basic residues at the P₁, P₂, and P₄ positions that mimic proprotein processing sites (Barr, 1991; Watanabe et al., 1995).

The inhibition of subtilisins by serpins is indistinguishable and, presumably, identical to inhibition of chymotrypsin family members. Therefore, whatever mechanism serpins use, it must account for inhibition of both the subtilisins and the chy-

motrypsins, two families that use similar catalytic mechanisms but contain completely unrelated scaffolds.

Materials and methods

Materials

DNA cellulose was from Worthington Biochemical Corp. Eglin c was a gift from Dr. Hans-Peter Schnebli (Ciba-Geigy, Basel). SCARL and PROK were obtained from two commercial sources, Sigma/Fluka and Worthington/Sigma, respectively. Reactigel was from Pierce. Bovine pancreatic trypsin inhibitor was from Boehringer Mannheim. Ostrich ovomucoid was a generous gift from Dr. Michael Laskowski (Purdue). All *p*-nitroanilide (*p*NA) substrates and 4-methylumbelliferyl *p*-(NNN-trimethyl-ammonium)cinnamate were from Sigma. Na¹²⁵I (17.4 Ci/mmol) was obtained from New England Nuclear (Boston, Massachusetts). Iodo-beads® were from Pierce (Rockford, Illinois).

Isolation and purification of serpins and proteinases

Serpins were isolated from human plasma as described previously (Mast et al., 1991). DNA affinity chromatography was used to further purify α_1 ACT (Rubin et al., 1990). All commercial preparations of subtilisins were further purified by cation exchange chromatography (Mono S) in 20 mM NaHOAc buffer containing 10 mM CaCl₂, eluted by 0–0.2 M NaHOAc gradient. Cat G and HNE were isolated from neutrophil granules by a modification of a standard method using affinity chromatography on immobilized bovine pancreatic trypsin inhibitor followed by cation exchange chromatography (Baugh & Travis, 1976). Immobilized ostrich ovomucoid third domain was used to further purify HNE after the cation exchange step in the standard protocol.

Radioiodination of subtilisin Carlsberg

SCARL was radio-iodinated by using Iodo-beads® according to instructions provided by the manufacturer (Pierce). Radiolabeled protein was separated from unincorporated iodine by gel filtration in 20 mM NaHOAc buffer, pH 4.5. Specific radioactivity was determined to be 6×10^4 cpm/ μ g.

Protein concentration determination

Total protein concentrations were determined based on absorbance at 280 nm and the molar absorption coefficients, which were calculated from the residue composition of Trp and Tyr according to the relationship previously described (Edelhoch, 1967).

PAGE

Protein samples were analyzed by nondenaturing PAGE or reduced SDS-PAGE in gels of 7% acrylamide or 5–15% linear acrylamide gradients as indicated. Samples that contained proteinases were treated with 1/3 volume of ice-cold 30% TCA to terminate the reaction. The precipitate was washed once with 10% TCA and twice with a 1/1 (v/v) ethanol–acetone mixture. The precipitate was air dried, and subjected to SDS-PAGE analysis after boiling for 5 min in sample buffer containing 1% SDS and 20 mM DTT. TUG gels were cast with a 0–8 M urea gradi-

ent in 7% acrylamide gels as previously described (Goldenberg, 1989; Mast et al., 1992).

Protein sequencing

Protein samples were run in PAGE, transferred to polyvinylidene difluoride membranes, which were stained briefly to detect protein (Matsudaira, 1987). Protein bands were excised from the membranes for N-terminal protein sequencing using an Applied Biosystems 476A sequencer.

Active titration of proteins

Chymotrypsin was active-site titrated using the fluorescent titrant and a Perkin-Elmer LS-50 spectrofluorometer (Jameson et al., 1973). Eglin c was standardized with the titrated chymotrypsin, and all other proteinases used in this study were active site titrated with the standardized eglin c.

The active concentrations of α_1 PI and α_1 ACT were determined by titrating them with standardized HNE, chymotrypsin, or cat G as appropriate (Salvesen & Nagase, 1989). All titrations were done at 37 °C in a microplate Reader (Molecular Devices) in 50 mM Tris buffer, pH 7.4, containing 0.1 M NaCl and 0.05% Nonidet P-40, and the appropriate substrates.

Inhibition assays

Chymotrypsin and subtilisins were titrated with inhibitors and residual activity measured normally using 0.5 mM suc-Gly-Gly-Phe-*p*NA when the enzyme concentrations exceeded 100 nM. This relatively high proteinase concentration was required to ensure complete inhibition in the 15-min time frame of the assay, and required the use of the relatively insensitive substrate. HNE was measured using 0.5 mM suc-Ala-Ala-Ala-*p*NA, and cat G was measured with 0.5 mM suc-Ala-Ala-Pro-Phe-*p*NA. For progress curve measurements of subtilisins at enzyme concentrations in the 10–100 pM range, a more sensitive substrate, 0.5 mM suc-Ala-Ala-Pro-Phe-*p*NA, was used. All proteinase/inhibitor assays were in 50 mM Tris buffer, pH 7.4 containing 0.1 M NaCl and 0.05% Nonidet P-40. SCARL and PROK assays also contained 10 mM CaCl₂.

Determination of association rate constant (k_{ass})

The association rate of subtilisins with serpins was measured by the progress curve method, where enzyme is incubated with 1.0 mM substrate and inhibitor simultaneously. Residual proteinase activity over time was determined by measuring substrate hydrolysis at 405 nm using a Molecular Devices *T*_{max} microplate reader operating in the kinetic mode. The first-order rate of inhibition (k_{obs}) was calculated from the following equation (Knight, 1986):

$$\ln([P]_{\infty} - [P]_t) = \ln[P]_{\infty} - k_{obs}t,$$

where $[P]_{\infty}$ is the product concentration at the end of the association reaction and $[P]_t$ is the product concentration at time *t*. The second-order rate (k_{ass}) was determined by allowing for the inhibitor concentration adjusted by the partition ratio (*I*), substrate concentration (*S*), and *K_m* at 37 °C, according to the relationship:

$$k_{ass} = (k_{obs}/I)(1 + S/K_m).$$

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