Location of the Protease-inhibitory Region of Secretory Leukocyte Protease Inhibitor*

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Secretory leukocyte protease inhibitor (SLPI) is a two-domain protein that inhibits a wide range of proteases including chymotrypsin, leukocyte elastase, and trypsin. Based on its homology to other protease inhibitors and on x-ray crystallography of an SLPI-chymotrypsin complex it has been proposed that the elastase and chymotrypsin-inhibitory site is in the COOH-terminal domain and that the trypsin-inhibitory site is in the NH-terminal domain.

We have prepared muteins of SLPI by site-directed mutagenesis of a synthetic gene for the protein, followed by expression in Escherichia coli. The protease-inhibitory activities of these muteins indicate that leucine 72 in the COOH-terminal domain is at the inhibitory site for elastase and chymotrypsin. Unexpectedly, our measurements indicate that the trypsin-inhibitory site is not in the NH-terminal domain. Instead they suggest that leucine 72 is also the inhibitory site for trypsin, even though the amino acid residues at the inhibitory sites of other trypsin inhibitors are almost always either lysine or arginine.

Two groups have recently reported the primary structure of a novel human protease inhibitor, secretory leukocyte protease inhibitor (SLPI)1 (1) and of a two-chain form of this inhibitor, human seminal plasma inhibitor 1 (2). This protease inhibitor is found in various secretory fluids including parotid secretions, bronchial, nasal, and cervical mucus, and seminal fluid (for reviews on this protein see Refs. 2, 3). The inhibitor forms complexes with a variety of proteolytic enzymes including the neutrophil proteases elastase and cathepsin G and the pancreatic proteases chymotrypsin and trypsin. The protein is of interest because it appears to be an important component of the antiprotease defense of tissues bathed by the secretory fluids. SLPI could be useful as a therapeutic in degenerative and inflammatory diseases that lead to proteolytic damage to these and other tissues.

The amino acid sequence of SLPI led to the prediction that the protein consists of two highly homologous domains of 53 and 54 amino acids (1, 4). This prediction was supported by the gene structure showing that the two domains are encoded on separate exons (5), and has recently been confirmed by x-ray crystallographic structure determination of recombinant SLPI (6). On the basis of a weak homology with the Kazal class of protease inhibitors (7), two regions of the protein were identified as being likely sites of interaction with proteases (1, 4, 6). One of these, present in the COOH-terminal domain of SLPI, was proposed to be the site interacting with chymotrypsin-like enzymes and elastase. This prediction of the chymotrypsin inhibitory site of SLPI has recently been confirmed by Grutter and colleagues (6) who have determined the structure of a crystal of the SLPI-chymotrypsin complex. The other region, present in the homologous position of the NH-terminal domain, has been proposed as the site of interaction with trypsin.

Numerous investigations of the interactions of proteases and their inhibitors have shown that these inhibitors bind to proteases in the same manner as substrates (7). As a consequence, proteases generally show a similar specificity for inhibitors and substrates, particularly in terms of the amino acid residue P1 of inhibitors/substrates that binds to subsite S1 of the enzyme. Based on this understanding, and a knowledge of the key amino acid residues that interact with the protease, it has been possible by elegant protein chemistry, and by site-directed mutagenesis techniques, to produce non-functional inhibitors and to target inhibitors to new enzymes (8–11). We reasoned that the unknown amino acid residues of SLPI that interact with elastase and trypsin could be identified by determining which residues, when changed, change the specificity of the inhibitor. We have, therefore, studied the properties of a number of variants of SLPI prepared by site-directed mutagenesis and expression of the gene for SLPI in Escherichia coli.

The results we have obtained provide independent confirmation of Grutter’s assignment of the chymotrypsin-inhibitory site of SLPI to residue Leu72 of the COOH-terminal domain. They also indicate that the same residue, Leu72, is the inhibitory site for leukocyte elastase. However, the results clearly indicate that Arg82, the residue homologous to Leu72 in the NH-terminal domain, is not the residue interacting with the S1 subsite of trypsin. Instead, they indicate that the trypsin-inhibitory site of SLPI is also residue Leu72, even though Leu is not a residue commonly thought to bind strongly to the S1 subsite of this enzyme.

MATERIALS AND METHODS

Expression Vector

The vector for expression of SLPI, pCJXI-2, was derived from pKK223-3 (Pharmacia I. K. R Biotechnology Inc.) by replacing the partial tetracycline-resistance gene with the complete pBR322-derived gene1 and by inserting an XhoI linker (New England Biolabs, catalog number 1030) into the unique PvuII site, and cloning the lacI gene EcoRI fragment from pMC9 (12) into the new XhoI site using EcoRI-XhoI adaptors.

SLPI Operon

The SLPI operon is an entirely synthetic fragment of DNA. Its sequence is based on: (a) the natural sequence of the ompA transla-

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1 The abbreviations used are: SLPI, secretory leukocyte protease inhibitor; MES, 4-morpholineethanesulfonic acid.

2 S. P. Eisenberg et al., manuscript in preparation.
tion-initiation region (13); (b) an efficient translation-coupler sequence (14, 15); and (c) the sequence of an SLPl gene as determined by reverse translation of the SLPl amino acid sequence (1). Codons were chosen to correspond to those used most abundantly by E. coli in highly expressed genes (16).

Details of the construction of the operon will be published elsewhere. Briefly, the operon was synthesized in five sections from oligonucleotides (synthesized on an Applied Biosystems 381A synthesizer and purified by polyacrylamide gel electrophoresis) which were phosphorylated, annealed, and cloned into M13 according to standard techniques. Once sequenced, each section was cut out of M13 using appropriate restriction enzymes and purified by polyacrylamide gel electrophoresis. The sections were then combined and recloned into M13 mp18. Several clones were sequenced, and one clone having the correct sequence, SLPlSynth-12, was chosen for mutagenesis. The complete sequence of the SLPl operon is given in Fig. 1.

Mutagenesis

Site-specific mutagenesis of SLPl was performed essentially as described by Kunkel (17) using the Mutagene in vitro mutagenesis kit purchased from Bio-Rad. Plaques were screened initially by hybridization using the mutagenic oligonucleotide as the probe (18), and those clones that were positive by hybridization were plaque-purified and sequenced using modified TT DNA polymerase (19), purchased as a kit from United States Biochemical Corp. Mutants having the desired sequence were grown in JM109 and M13 RF DNA was prepared (20).

Preparation of SLPl Variants

The EcoRI-PstI fragment carrying the SLPl gene was isolated from the M13 RF DNA as described above and inserted into pCJXI-9 at the corresponding restriction sites. Competent JM109 cells were transformed, and a tetracycline-resistant colony was picked, purified, and grown at 37 °C in LB broth containing 10 μg of tetracycline/ml to a density of 2 × 10⁶ cells/ml. At this point, the culture was induced with 1 mM isopropyl-β-D-thiogalactopyranoside, and after 3 h of continued incubation the culture was cooled and the cells were pelleted, washed with 0.05 M Tris-HCl (pH 7), repelleted, and frozen.

To prepare SLPl and its variants the cells were treated as follows. The pellet was thawed and suspended in 100 mM Tris-HCl (pH 7.5) containing 8 mM urea, 4 mM Na₂EDTA, 50 mM 2-mercaptoethanol and passed twice through a French press at 12,000 p.s.i. to lyse the cells and shear the DNA. The lysate was then adsorbed to Sephadex SP-C25 (Pharmacia LKB Biotechnology Inc.) and was eluted with 0.4 M guanidine HCl, 100 mM Tris-HCl (pH 7.5), and 50 mM 2-mercaptoethanol. An equal volume of 6 M guanidine HCl was added to the SP-C25 eluant followed by sequential additions of diithothreitol to 3.5 M, and oxidized glutathione to 1.5 M. The mixture was then diluted with 4.5 volumes of 50 mM Tris-HCl (pH 10.7) and cytostatin was added to a final concentration of 3 mM. After an overnight incubation at 25 °C the SLPl-refolding mixture was adjusted to pH 6.0 and diluted with 20 mM MES (pH 6.0) to lower the guanidine HCl concentration to below 150 mM. It was applied to a Mono S HR 5/5 column (Pharmacia LKB Biotechnology Inc.) equilibrated with 20 mM MES (pH 6.0) and was eluted with a 1%/min NaCl gradient in the same buffer. SLPl elutes at 350 mM NaCl using these chromatographic conditions.

SLPl concentrations were determined either by Bradford assays (Bio-Rad) or spectrophotometrically (4,21) in 20 mM MES (pH 6.0) and 350 mM NaCl. The activity of SLPl and SLPl variants was determined by measuring their ability to inhibit chymotrypsin-catalyzed hydrolysis of succinyl-Ala-Ala-Pro_Phe nitroanilide (21), elastase-catalyzed hydrolysis of methoxysuccinyl-Ala-Ala-Pro-Val_Phe nitroanilide (22), or trypsin-catalyzed hydrolysis of tosyl-Gly-Pro_Lys_Phe nitroanilide (Boehringer Mannheim). As a measure of the affinity of chymotrypsin, elastase, and trypsin for the various SLPl mutants, dissociation constants were determined essentially as described previously (1).

Succinylation of Proteins—Solid succinic anhydride was added to a 0.15 mg/ml solution of SLPl, SLPl-Lys, or bovine pancreatic trypsin inhibitor (Calbiochem) to achieve a final concentration of 1.5 M in 1 M sodium borate (pH 9.0). The pH was monitored during the 1-h reaction period and, when necessary, aqueous 5 M NaOH was added to maintain the pH above 8 (23).

Protein Sequencing—Protein sequencing was performed on an Applied Biosystems model 470A sequenator followed by HPLC identification of phenylthiohydantoins (24).

RESULTS

Preparation of SLPl and SLPl Variants

SLPl protein is conveniently expressed as part of a two-cistron operon (see “Materials and Methods”) such that translation of the SLPl gene is coupled to translation of an efficiently expressed upstream gene, in this case the first 10 codons of the E. coli ompA gene. Transcription of this operon from the Tac promoter is very tightly regulated due to the presence of high levels of lac repressor expressed from the lacI gene found on the F factor and the wild-type lacI gene carried on the plasmid. Thus, very low levels of SLPl are present in the uninduced state. However, when the culture is induced with isopropyl-β-D-thiogalactopyranoside, SLPl is normally expressed to a level of 5–10% of total cellular protein, making purification, activation, and assaying the protein relatively straightforward. We have shown, in the case of the parent protein, that the NH₂-terminal methionine residue is removed by processing systems in E. coli, and that the recombinant protein has a sequence identical to that of human SLPl.

Site-specific mutagenesis of the SLPl gene was performed using standard methods. A list of oligonucleotides and the corresponding amino acid changes is shown in Table I. The efficiency of mutagenesis was usually between 30 and 50%. SLPl and SLPl variants can be readily purified from lysed cells by cation-exchange chromatography and can be induced to fold to an active conformation by reduction and denaturation followed by oxidation and dilution of the denaturant, and
Interestingly, when Leu7' of SLPI is replaced by Gly the protein loses most of its ability to inhibit chymotrypsin and some of its ability to inhibit elastase. In residues interacting with the S1 subsites of elastase and chymotrypsin, they are also consistent with the alternative hypothesis that the Gly7' variant of SLPI is unable to fold to an active form under the same conditions as the natural protein.

To explore this possibility we compared other properties of the Gly7' variant of SLPI with the natural protein. Active and folded forms of SLPI elute at 25 and 30% acetonitrile, respectively, on the C8 column used for reversed-phase chromatography and at 350 mM and 370 mM NaCl, respectively, on the Mono S column used for cation-exchange chromatography. Despite the sensitivity of these techniques to conformation, we were unable to demonstrate any chromatographic differences between SLPI and SLPI-Gly7' on either C8 or Mono S columns.

Another remarkable feature of the folded form of SLPI is its resistance to proteolysis by a number of proteases that are not inhibited by this protein. Since denatured SLPI is an excellent substrate for these proteases, the resistance to proteolysis of the native form of this protein is likely to be a measure of its conformational integrity. We find that SLPI-Gly7' shares the marked resistance of SLPI to digestion with pepsin and with thermolysin (3).

Of the many pepsin-cleavable bonds of SLPI, only one (Leu72-Met73) is subject to pepsin cleavage in its native conformation since NH2-terminal sequencing of the digested protein shows the presence of two sequences starting at Ser71 and Met73. This same resistance to proteolysis is seen with SLPI-Gly7', with the added twist that even the bond between residues 72 and 73 is now resistant to pepsin, doubtless because Gly-X bonds are not substrates for this enzyme. Similarly, of the many thermolysin cleavable bonds in SLPI, only two (Cys18-Leu19 and Met72-Leu73) are subject to thermolysin cleavage in the native protein. SLPI-Gly7' shares this resistance to proteolysis by thermolysin in that only the Cys18-Leu19 bond is cleaved by this enzyme. The absence of one cleavage, characteristic of the natural molecule, is probably attributable to the amino acid substitution (Gly7') close to the prospective site of cleavage. However, the absence of hydrolysis at all other sites is likely attributable, as with the native molecule, to a characteristic tertiary structure that restricts access of the proteases to what would otherwise be susceptible bonds. We conclude that the variant SLPI-Gly7' has a similar structure to natural SLPI. Its reduced ability to inhibit chymotrypsin and elastase is likely, therefore, a consequence of the fact that Leu7' of the natural inhibitor binds to the S1 subsite of these proteases and contributes strongly to the binding energy of the inhibitor to the enzyme.

To confirm the hypothesis that residue Leu7' binds to the active site of leukocyte elastase we made a further variant of SLPI with Phe substituted for Leu7'. It is known that leukocyte elastase cannot hydrolyze peptide bonds COOH-terminal to Met and Leu are often good substrates of chymotrypsin and leukocyte elastase, whereas analogous peptide bonds COOH-terminal to Gly are very poor substrates of these enzymes (26, 27). Interestingly, when Leu7' of SLPI is replaced by Gly the protein loses most of its ability to inhibit chymotrypsin and some of its ability to inhibit elastase. In contrast, when Met73 or Leu7' of SLPI is replaced by Gly the protein retains some of its activity against chymotrypsin and has close to full activity against elastase (Table II).

Although these findings are consistent with Leu7' being the residue interacting with the S1 subsite of elastase and chymotrypsin, they are also consistent with the alternative hypothesis that the Gly7' variant of SLPI is unable to fold to an active form under the same conditions as the natural protein. To explore this possibility we compared other properties of the mutant and natural proteins. By several tests SLPI-Gly7' and the natural protein appear to have quite similar conformations.

We first explored the chromatographic properties of SLPI and SLPI-Gly7' on reversed-phase and cation-exchange HPLC. In other experiments we have shown that these properties are quite sensitive to the conformation of SLPI. Active and unfolded forms of SLPI elute at 25 and 30% acetonitrile, respectively, on the C8 column used for reversed-phase chromatography and at 350 mM and 370 mM NaCl, respectively, on the Mono S column used for cation-exchange chromatography. Despite the sensitivity of these techniques to conformation, we were unable to demonstrate any chromatographic differences between SLPI and SLPI-Gly7' on either C8 or Mono S columns.
to Phe but that these bonds can be hydrolyzed by chymotrypsin (28). In accord with the idea that Leu$^{72}$ of SLPI binds to the S1 subsite of elastase we observed that SLPI-Phe$^{72}$ is a weak inhibitor of this enzyme. However, SLPI-Phe$^{72}$ does inhibit chymotrypsin, indicating that the protein has adopted the correct conformation. The latter result provides a compelling argument that the substitution of Phe for Leu has specifically altered the ability of the protein to inhibit elastase by changing a residue that is critical for the interaction with that enzyme.

**Trypsin—Peptide bonds COOH-terminal to Arg** are generally excellent substrates of trypsin whereas analogous peptide bonds COOH-terminal to Gly are very poor substrates for this enzyme. However, when Arg$^{70}$ of SLPI is replaced with Gly the resulting protein is still active as an inhibitor of trypsin and the $K_d$ of the complex of trypsin with SLPI-Gly$^{70}$ is almost indistinguishable from that of natural SLPI (Table II). That this variant still inhibits trypsin argues strongly against the view that Arg$^{70}$ is the residue of SLPI binding to the S1 subsite of trypsin. Also consistent with this view are our findings that replacement of Arg$^{70}$ by Val or Met gives SLPI variants that have activity against trypsin. We conclude that Arg$^{70}$ is not involved in interaction between SLPI and trypsin.

Earlier results from our laboratory have shown that the COOH-terminal domain of SLPI expressed from a partial gene in yeast has full activity against elastase and chymotrypsin but has little or no activity against trypsin (29), leading us to discount the possibility that the residues in this domain could play a significant role in the antitrypsin activity of the inhibitor. More recently, we have split the two domains of SLPI by treatment with formic acid and again found undetectable antitrypsin activity in the COOH-terminal fragment. However, when we tested the antitrypsin activity of the NH$_2$-terminal domain produced by formic acid cleavage we found that it was also inactive against trypsin, indicating that the antitrypsin activity of SLPI is a property of the whole molecule, and that the antitrypsin site could well reside in the COOH-terminal domain. We therefore turned our attention to the variants in the COOH-terminal domain in our search for the trypsin-inhibitory site.

Neither SLPI-Gly$^{72}$ nor SLPI-Gly$^{72}$ inhibit trypsin (Table II) indicating that residue Leu$^{72}$ or Met$^{73}$ might occupy the S1 subsite of trypsin in SLPI-trypsin complexes. This would be surprising because neither of these residues have the basic side chain expected of the P1 residues of normal trypsin substrates and inhibitors. To investigate further the possibility that either Leu$^{72}$ or Met$^{73}$ of SLPI may be the P1 residue in SLPI-trypsin complexes we changed residue 72 to Val, since it had been noted earlier that changing the P1 residue of α1 protease inhibitor to Val reduced the ability of this protein to inhibit trypsin (30). SLPI-Val$^{72}$ is a poor trypsin inhibitor but appears to have the correct conformation since it is an excellent elastase inhibitor (Table II). This suggests that Leu$^{72}$ is the trypsin-inhibitory active site of SLPI.

To obtain further evidence for this possibility we substituted a Lys residue and an Arg residue for the Leu at position 72. Since peptides with P1 Lys or Arg residues bind much more strongly to trypsin than peptides without a basic group at this position, we would expect these variants to be considerably stronger inhibitors of trypsin than the natural molecule if this residue binds to the S1 subsite of trypsin. We found that the dissociation constants of both SLPI-Lys$^{72}$-trypsin and SLPI-Arg$^{72}$-trypsin complexes are at least 1000-fold lower than that of the natural SLPI-trypsin complex (Table II). We conclude that either Leu$^{72}$ or SLPI is the trypsin-inhibitory active site, or that some residue other than Leu$^{72}$ could be the original trypsin-inhibitory site and changing Leu$^{72}$ to Lys or Arg has created a new antitrypsin site which has masked the original.

It is possible to distinguish between these two possibilities by succinylating SLPI-Lys$^{72}$. Succinylation of the P1 lysine residue of a protease inhibitor should reduce its ability to bind to the trypsin-active site. As a control for this experiment we showed that the activity of bovine pancreatic trypsin inhibitor, whose active site is known to be Lys$^{30}$ (31), is destroyed by succinylation (Table III). The antitrypsin activity of wild type SLPI is only slightly affected by succinylation; therefore, we reasoned that if SLPI-Lys$^{72}$ has a completely novel antitrypsin site succinylation should inactivate this site and reveal the continued presence of the old antitrypsin site. If, on the other hand, the substitution of Lys for Leu$^{72}$ has simply improved the old antitrypsin site, succinylation should abolish the antitrypsin activity of SLPI-Lys$^{72}$ altogether. The data in Table III show that succinylated SLPI-Lys$^{72}$ has no significant trypsin-inhibitory activity, thus favoring the hypothesis that residue 72 of SLPI is the trypsin-inhibitory active site.

**DISCUSSION**

The specificities of proteases for substrates and inhibitors are known to show extensive similarities. At the structural level, this result is explicable in terms of the similar binding modes of substrates and inhibitors. The substrate specificity of the three enzymes studied here has been established by several groups. Briefly summarized, these investigations indicate that trypsin is highly specific for peptide bonds COOH-terminal to arginine and lysine, chymotrypsin is specific for bonds COOH-terminal to large hydrophobic and especially aromatic amino acid residues, and leukocyte elastase is specific for bonds COOH-terminal to amino acids with moderately large hydrophobic side chains. Although these substrate specificities are suggestive in pointing to which residues of SLPI might bind to the S1 subsites of these enzymes, they are insufficient to unambiguously define the region of SLPI interacting with these proteases. To distinguish those regions where the polypeptide chain of SLPI accidentally conforms to the specificity of the protease from those where the match is vital to the function of the inhibitor, we have systematically altered the sequence of the inhibitor, and determined how these changes affect its specificity.

It is possible to envision three extreme results of this kind of investigation. An amino acid substitution that would greatly reduce the ability of a peptide to be a substrate but that has no effect on the inhibitory ability of SLPI should indicate unambiguously that the residue substituted does not play a vital role in SLPI interaction with the protease. In contrast, an amino acid substitution that would reduce the

### Table III

| Protein | Trypsin $K_i$ (nM) | Chymotrypsin $K_i$ (nM) |
|---------|-------------------|------------------------|
| BPTI$^a$ | $<$0.001          | $>$100                 |
| BPTI$^b$ | $>$1000          | $>$100                 |
| SLPI    | 3.0              | 0.4                    |
| SLPI$^b$ | 55.0             | 0.8                    |
| SLPI-Lys$^{72}$ | $<$0.003 | $>$100                 |
| SLPI-Lys$^{72}$ | $>$1000 | $>$100                 |

$^a$ BPTI, bovine pancreatic trypsin inhibitor.
$^b$ Succinic anhydride-treated protein.
$^c$ Natural sequence (Leu$^{72}$).

- Kinetic dissociation constants of succinic anhydride-treated proteins.
ability of a peptide to act as a substrate and which also reduces the inhibitory activity of SLPI supports, but does not conclusively prove, that the residue altered plays a role in the enzyme-inhibitor interaction: an amino acid substitution that prevents the protease inhibitor reaching its active conformation will also produce this result. To confirm that the residue changed has some direct role in the enzyme-inhibitor interaction, some independent evidence that the defective inhibitor has reached the “active” conformation is required. Finally, an amino acid substitution that would improve the ability of a peptide to act as a substrate and that improves the activity of SLPI strongly supports the hypothesis that the residue changed plays an important role in the enzyme-inhibitor interaction.

By the criteria described above we believe that we have proved that residue Leu72 of SLPI binds to the S1 subsite of leukocyte elastase. The evidence in favor of this hypothesis is that a change from Leu to Gly or Phe or Lys at this position reduces the protein’s ability to act as an elastase inhibitor and that by several other criteria, including in the latter two cases a continued ability to inhibit related proteases (chymotrypsin and trypsin, respectively), the variant inhibitor appears to be in the native conformation. The change from Leu to Gly or Phe in an elastase substrate is known to abolish the ability of a peptide to act as an elastase substrate (27).

By the criteria we believe that we have also located the chymotrypsin-active site of SLPI at Leu72. In favor of this hypothesis is the observation that SLPI-Gly72, SLPI-Lys72, and SLPI-Arg72 are poor chymotrypsin inhibitors, even though their conformations appear to be similar to that of the natural protein. This evidence is particularly strong for SLPI-Lys72 and SLPI-Arg72 because these proteins retain the ability of SLPI to inhibit trypsin. Residues Met73 and Leu74, two other candidates for binding to the chymotrypsin-active site, are unlikely to bind to subsite S1 because SLPI-Gly720 and SLPI-Gly74 are still chymotrypsin inhibitors (relative to SLPI-Gly72) although they are poorer than wild type SLPI. The reduced affinity of SLPI-Gly72 for chymotrypsin is easily explained in terms of its binding to subsite S1’ of chymotrypsin since this subsite also shows some specificity for hydrophobic residues (27). Our work, therefore, confirms the conclusion of Grutter et al. (6) from x-ray crystallography of a complex of SIPI with chymotrypsin.

By the criteria described above we believe that we have conclusively demonstrated that Arg70 is not the trypsin-inhibitory site of SIPI. The best candidate for this residue appears to be Leu72. In favor of this assignment we note that SLPI-Gly720, SLPI-Met720, and SLPI-Val720 are all good inhibitors of trypsin, whereas SLPI-Gly72 and SLPI-Val72 are not good inhibitors of trypsin even though, as shown above, the latter two proteins appear to have the correct conformation. SLPI-Gly72 is also not a strong trypsin inhibitor suggesting that Met-73 could be the trypsin-inhibitory site.

The strongest evidence in favor of the hypothesis that Leu72 is the trypsin-inhibitory site is the fact that SLPI-Lys72 and SLPI-Arg72 are much stronger inhibitors of trypsin than native SIPI. In addition, the trypsin-inhibitory activity of SLPI-Lys72, unlike that of SLPI itself, is fully inactivated by treatment with succinic anhydride. Finally, we note that Kramps et al. (32), have previously proposed that the antielastase and antitrypsin sites of SIPI might be the same on the basis of their similar susceptibility to oxidative inactivation.

The finding that a nonbasic amino acid residue can occupy subsite S1 of the active site of trypsin, although surprising, is not entirely without precedent. The α-1 protease inhibitor is a weak inhibitor of trypsin, even though a methionine residue is thought to be the P1 amino acid of this inhibitor. Certainly, substitution of this Met by Arg improves the affinity of α-1 protease inhibitor for trypsin (33). This result reinforces the conclusion, reached many years ago on the basis of studies of the substrate specificity of these enzymes, that contacts between the enzyme and its ligand, apart from those in the S1 subsite, may make important contributions to enzyme-ligand affinities. The novel feature of the interaction between SLPI and trypsin is that, on the basis of the apparent lack of activity of the COOH-terminal domain of SLPI against trypsin, some of these contacts involve residues in a different domain of the inhibitor.

Our results indicate that the antielastase, antichymotrypsin, and antitrypsin sites of SLPI all reside in the COOH-terminal domain of the molecule and raise the question of the role of the NH2-terminal domain of this protein. Clearly the NH2-terminal domain could be present simply to provide some extra binding energy, as it does in the case of trypsin. However, the distribution of trypsin and SLPI virtually ensure that this particular protease-antiprotease complex has little biological significance. It is possible that the NH2-terminal domain is a protease inhibitor whose target is yet to be found, although we note that the small changes necessary to maximize the homology between the two domains (1) may have dramatic consequences for the conformation of the “active site” loop of domain 1. Alternatively, it may even be that domain 1, which was once an active inhibitor itself, now serves a negative regulatory function and restricts SLPI’s action to a few proteases that are not part of the vital proteolytic systems of the body.

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Inhibitory Active Site of SLPI

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