The primary elastase inhibitor (elastasin) and trypsin inhibitor (contrapsin) in the goat are serpins related to human α_1 -anti-chymotrypsin

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Two primary serine proteinase inhibitors in goat plasma have been isolated and characterized. The N-terminal sequence analysis of the purified proteins revealed that they are closely related to each other and are highly homologous to human α_1 -anti-chymotrypsin rather than α_1 -proteinase inhibitor. However, despite structural similarities the inhibitory specificity of the goat inhibitors differed from each other and from that of anti-chymotrypsin. In contrast with human anti-chymotrypsin, one of the goat inhibitors was shown to be a strong and specific inhibitor of trypsin ($k_{\text{ass.}} = 1.9 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$), whereas the other was an efficient inhibitor of neutrophil elastase ($k_{\text{ass.}} = 1.5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$). Differences in the inhibitory specificity of each protein could readily be attributed to the amino acid

sequence within the reactive site region. The trypsin inhibitor with an assumed arginine residue at the P_1 position of the reactive-site peptide bond is referred to as 'contrapsin', and indicates that the occurrence of contrapsins is not restricted to rodents. In contrast, the inhibitory specificity, resistance to oxidative and proteolytic inactivation and the presence of a P_1 leucine residue in the elastase inhibitor is unique among inhibitory serpins that have been characterized to date. Because this serpin is apparently the major elastase inhibitor in goat plasma, it is likely to be involved in the control of goat neutrophil elastase. Therefore, we suggest the name 'elastasin', and extend it to any other anti-chymotrypsin related serpins possessing neutrophil-elastase-inhibitory activity.

INTRODUCTION

α_1 -Proteinase inhibitor (α_1 -PI) and α_1 -anti-chymotrypsin (α_1 -ACHY) are the two most abundant serine proteinase inhibitors (serpins) found in human plasma (Travis and Salvesen, 1983). The physiological function of α_1 -PI is well defined and, although it inactivates numerous mammalian serine proteinases, its primary role is in the regulation of neutrophil elastase. In inhibitory deficiency states, free elastase can cause the abnormal degradation of connective tissue, ultimately resulting in various disease states, and especially emphysema (Perlmutter and Pierce, 1989). Although control of this enzyme by α_1 -PI is quite rigid, it can be overcome by inactivation of the inhibitor through either chemical (cigarette smoke) (Janoff and Carp, 1977) or enzymic (neutrophil myeloperoxidase) (Matheson et al., 1979) oxidation of its reactive-site methionine residue. It has been suggested that this mechanism, assisted by proteolytic inactivation of the inhibitor (Desrochers et al., 1991, 1992), is involved in connective-tissue diseases in individuals with normal α_1 -PI levels. On the other hand, it is believed that oxidative and proteolytic mechanisms for serpin inactivation are utilized in normal tissue modelling (Carrell and Travis, 1985; Carrell and Owen, 1985).

By contrast with α_1 -PI, the precise biological role of α_1 -ACHY is somewhat obscure, although it has been implicated in a wide variety of physiological processes *in vivo*. In addition to regulating cathepsin G activity (Beatty et al., 1980) and possibly other chymotrypsin-like proteinases (Davril et al., 1987; Schechter et al., 1989; Christensson et al., 1990; Nelson and Siman, 1990), anti-chymotrypsin modulates the cellular functions of neutrophils (Kilpatrick et al., 1991) and lymphocytes (Hudig et al., 1981; Matsumoto et al., 1981), inhibits platelet-activating-factor synthesis (Camussi et al., 1988), controls certain parasitic invasion mechanisms (McKerrow, 1988), and it may regulate the processing of β -amyloid in Alzheimer's disease and Down's syn-

drome (Abraham et al., 1988). A familial form of α_1 -ACHY deficiency has been described and, although the heterozygote frequency in Sweden is 0.6 %, no homozygote has been reported (Lindmark, 1990). This strongly indicates that such a genotype may be incompatible with life, and further underscores the physiological indispensability of α_1 -ACHY.

α_1 -PI occurs in all mammalian species so far examined, and in many cases the inhibitor had been purified and fully characterized and/or its primary structure inferred from cDNA analysis (Patterson, 1991). Much less information is available on α_1 -ACHY-related inhibitors from other animal plasmas, although such data could prove useful in understanding α_1 -ACHY function in man. Several years ago Takahara and Sinohara (1982) described a trypsin inhibitor in mouse plasma which they named 'contrapsin' (contra trypsin), which was later shown to be highly homologous to human α_1 -ACHY (Hill et al., 1984). To date, several form of contrapsins have been identified in the plasma of guinea pigs (Suzuki et al., 1991), rats (Pages et al., 1990; Ohkubo et al., 1991a) and mice (Ohkubo et al., 1991b), but not in other mammals, leading to the suggestion that, like murinoglobulin, the occurrence of contrapsin is limited to rodents (Suzuki et al., 1991). In the present paper, however, we describe a detailed characterization of two major goat plasma proteinase inhibitors which show some structural homology to human α_1 -ACHY, albeit with an entirely different inhibitory specificity. One of these inhibits trypsin-like proteinases and is categorized as goat contrapsin, whereas the second one, an efficient neutrophil elastase inhibitor, is referred to as 'elastasin' (elastase inhibitor) to underline its unique specificity and structural relationship.

MATERIALS AND METHODS

Materials

MeO-Suc-Ala-Ala-Pro-Val-pNA (Suc, succinyl; pNA, *p*-nitro-

Abbreviations used: α_1 -PI, α_1 -proteinase inhibitor; α_1 -ACHY, α_1 -anti-chymotrypsin.

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anilide), Suc-Ala-Ala-Pro-Phe-pNA, Bz-Arg-pNA (Bz, benzoyl), Bz-Ile-Glu-Gly-Arg-pNA, di-isopropyl fluorophosphate, *N*-chlorosuccinimide, papain, thermolysin, bovine α -chymotrypsin, pig pancreatic elastase (type III), pig trypsin, Reactive Red 120-agarose, Reactive Green 5-agarose, concanavalin A-Sepharose, octyl-Sepharose, phenyl-Sepharose and iminodiacetic acid-Sepharose were from Sigma. EAH-Sepharose 4B was purchased from Pharmacia Biotech (Piscataway, NJ, U.S.A.). Trypsin and α -chymotrypsin were coupled with EAH-Sepharose 4B according to the manufacturer's recommendation. *Staphylococcus aureus* metalloproteinase (Drapeau, 1978), human neutrophil elastase and cathepsin G (Baugh and Travis, 1978), human cationic trypsin (Johnson and Travis, 1976), and human plasma α_1 -anti-chymotrypsin (Dubin et al., 1990) were isolated as described previously. Plasmin was prepared by activation of human plasminogen (Brockway and Castellino, 1982). *Pseudomonas aeruginosa* elastase, *Serratia marcescens* 56 kDa metalloproteinases, and human α -thrombin, were kindly given by Dr. J. Powers (Georgia Institute of Technology, Atlanta, GA, U.S.A.) and Dr. J. Fenton (New York State Health Department, Albany, NY, U.S.A.) respectively.

Purification of goat contrapsin and elastasin

All of the steps described below were performed at +4 °C. The purification scheme was based essentially on that of Pannell et al. (1974). Goat plasma (250 ml) was precipitated between 0.5 and 0.8 (NH₄)₂SO₄ saturation, dissolved in 25 ml of 0.03 M sodium phosphate, pH 6.7, and dialysed against several changes of the same buffer. The dialysed fraction (50 ml) was then applied to a column of Cibacron Blue-Sepharose (3.5 cm × 50 cm), equilibrated with the phosphate buffer. Trypsin- and elastase-inhibitory activities were eluted in the void volume, and this fraction was loaded directly on to a column (1.5 cm × 30 cm) of DE-52 DEAE-cellulose (Whatman), equilibrated with the phosphate buffer, pH 6.5. After passage of unretarded protein through the column, a linear NaCl gradient (1200 ml total) was initiated from 0 to 0.5 M NaCl. Trypsin- and elastase-inhibitor activities were found in the protein peak eluted between 0.09 and 0.1 M NaCl, and these activities were pooled. After increasing the pH to 8.0 by addition of 0.4 M Na₂HPO₄, the inhibitor-containing fraction was dialysed against 0.05 M Tris/HCl, pH 8.0, and rechromatographed on a column of DE-52 DEAE-cellulose (1.3 cm × 16 cm), equilibrated with 0.05 M Tris/HCl, pH 8.0. A linear gradient from 0 to 0.2 M NaCl (800 ml total) was initiated, and inhibitory activity, obtained in a single peak, was eluted at 0.11–0.12 M. At this stage the isolated protein was found to consist of a mixture of two closely related inhibitors of trypsin and elastase. Further efforts to separate these inhibitors by gel filtration on Sephadex G-100 (2 cm × 120 cm column, equilibrated with 0.1 M Tris/HCl/0.5 M NaCl, pH 7.5), affinity chromatography on dye resins (Reactive Red 120 and Reactive Green 5), lectin resin (concanavalin A), hydrophobic resins (butyl- and phenyl-Sepharose) and zinc-chelate chromatography (iminodiacetic acid) failed to yield satisfactory separations of anti-elastase and anti-trypsin activities. Therefore, final purification of each inhibitor was achieved by negative affinity chromatography on either a trypsin-Sepharose or chymotrypsin-Sepharose column (1.0 cm × 10 cm) in 0.05 M Tris/HCl, pH 8.0, followed by ion-exchange chromatography of the flow-through on the Mono Q f.p.l.c. column, equilibrated with 0.02 M Tris/HCl, pH 8.0. A linear NaCl gradient was developed from 0.1 to 0.3 M NaCl, and inhibitory activity was eluted (1 ml/min) at a salt concentration of about 0.2 M.

Sequence analysis

Automated Edman degradation of goat inhibitors alone, in complex with proteinases, or after proteolytic inactivation was carried out in an Applied Biosystems model 477A sequencer with on-line phenylthiohydantoin analysis using an Applied Biosystems model 120A h.p.l.c. apparatus.

Active-site titration

All enzymes utilized in kinetic studies were active-site titrated either with burst titrants (trypsin, chymotrypsin, plasmin, thrombin) (Keszdy and Kaiser, 1970) or by titration against α_1 -PI (human neutrophil elastase, cathepsin G), which was used as a secondary standard. Active-site titration of α_1 -PI was made against a standardized solution of pig trypsin.

Formation of contrapsin and elastasin complexes with target proteinases

Contrapsin (420 pmol) and pig trypsin (410 pmol), or elastasin (350 pmol) and human neutrophil elastase (350 pmol), all in 0.1 M NH₄HCO₃, pH 8.0, were incubated for 60 s at room temperature. The samples were then transferred directly to the sequenator and dried under vacuum before initiation of the program. Controls containing di-isopropyl fluorophosphate-treated pig trypsin or neutrophil elastase, the inhibitors, or equimolar mixtures of each, were also subjected to sequence analysis.

Limited proteolysis of goat inhibitors

The two purified goat inhibitors and human α_1 -ACHY were modified by limited proteolysis with *P. aeruginosa* elastase, *S. marcescens* 56 kDa metalloproteinase, *Staph. aureus* metalloproteinase, thermolysin, human neutrophil elastase or clostripain. Usually, a given inhibitor (15 μ M) and the bacterial proteinase (or human neutrophil elastase for goat contrapsin and α_1 -ACHY) were incubated at an inhibitor:enzyme molar ratio between 1:1 and 10000:1 in buffers suitable for a given enzyme. At various time intervals, samples were removed and tested for inhibitory activity toward pig trypsin, neutrophil elastase or cathepsin G, by addition of an equimolar amount of enzyme. After 3 min at room temperature the mixture was assayed for residual enzyme activity by using MeO-Suc-Ala-Ala-Pro-Val-pNA (elastase), BAPNA (trypsin) or Suc-Ala-Ala-Pro-Phe-pNA (cathepsin G), respectively. The effectiveness of inhibitor cleavage was confirmed by SDS/PAGE (Wyckoff et al., 1977), using a linear 8–20 % (w/v) acrylamide gradient. When inhibitory activity fell to about 10 % of control, the reaction was stopped by separation of modified inhibitor from proteinases on a Mono Q f.p.l.c. column, as described above. Fractions containing inactive inhibitor were freeze-dried, redissolved in deionized water, desalted by passage through Sephadex G-25 (Pharmacia) (0.8 cm × 5 cm column), and applied for sequencing.

Measurement of association rates

The second-order association rate constant for each goat inhibitor with a variety of proteinases was determined by the method of Bieth (1980). Equimolar mixtures of enzyme and inhibitor (240 nM for plasmin, 180 nM for pig pancreatic elastase and cathepsin G, and 10 nM for human neutrophil elastase and chymotrypsin, based on the active-site-titrated activities of each protein) were incubated in a total volume of 1.0 ml of 0.2 M

Tris/HCl, pH 8.0, for increasing time periods at room temperature. Residual enzyme activity was then measured by the addition of a saturating amount (final concn. 1 mM) of a specific proteinase substrate.

Oxidation of goat inhibitors

In order to determine the effect of oxidation on inhibitory activity each protein at a concentration of 50 μ M was incubated with a 30-fold molar excess of *N*-chlorosuccinimide (1.5 mM) at room temperature for 1 h in 0.1 M Tris/HCl, pH 8.0. Excess reagent was removed by dialysis, and second-order association rate constants were measured with specific proteinases, as described above.

RESULTS

Properties of goat inhibitors

The initial purification of goat plasma proteinase inhibitor consisted of $(\text{NH}_4)_2\text{SO}_4$ fractionation, affinity chromatography on Cibacron Blue–Sephacel and two subsequent anion-exchange-chromatography separations on DE-52 DEAE-cellulose, at pH 6.5 and pH 8.0. This procedure gave a yield of 70–90 mg of purified protein from 250 ml of plasma. However, SDS/PAGE analysis at this stage of purification always gave two protein bands, of apparent M_r 58 000 and 110 000 (Figure 1, lane a). Nevertheless, gel-filtration chromatography on Sephadex G-100 gave one symmetrical peak of protein and inhibitory activity of M_r 60 000. Moreover, SDS/PAGE of samples from ascending and descending shoulders of the peak revealed exactly the same pattern of protein bands as those before gel filtration. This indicates that the upper band might be a dimer formed in the presence of SDS. Indeed, SDS-induced dimerization is a feature of mouse contrapsin (Takahara and Sinohara, 1982) and could be avoided by protein carboxyamidomethylation before SDS/PAGE (results not shown).

Titration of the inhibitor obtained after gel filtration with trypsin and human neutrophil elastase gave unusual results

Figure 1 SDS/PAGE of goat trypsin and elastase inhibitors

Lanes: (a) 20 μ g of protein, the trypsin and elastase inhibitor mixture obtained after gel filtration; (b) 10 μ g of elastase inhibitor preincubated with *Staph. aureus* metalloproteinase; (c) 10 μ g of the protein from trypsin–Sephacel column flow-through; (d) 10 μ g of elastase inhibitor purified on a Mono Q f.p.l.c. column from the trypsin–Sephacel flow-through; (e) 5 μ g of proteolytically modified trypsin inhibitor (non-active peak after Mono Q f.p.l.c. chromatography of trypsin–Sephacel column); (f) 10 μ g of trypsin inhibitor partially inactivated by incubation with clostripain (2 min at 500:1 inhibitor:proteinase ratio); (g) molecular-mass markers; (h) 20 μ g of the final preparation of trypsin inhibitor; (i) inhibitor mixture preincubated with *P. aeruginosa* elastase at molar ratio 20:1 for 120 min; (j) inhibitor mixture preincubated with clostripain at molar ratio 500:1 for 120 min; (k) elastase inhibitor inactivated by *P. aeruginosa* elastase; (l) trypsin inhibitor preincubated with clostripain at molar ratio 500:1 for 20 min.

Figure 2 Titration of goat inhibitors with human neutrophil elastase and pig trypsin

The increasing amount of goat inhibitors mixture (the sample after gel filtration, 2.55 mg/ml) alone (●), or preincubated with 2-fold molar excess of trypsin over trypsin-inhibitory capacity (○), or preincubated with 2-fold molar excess of elastase over elastase-inhibitory capacity of the sample (△), or with saturated elastase inhibitory capacity (▲), were incubated in 0.99 ml of 0.2 M Tris/HCl, pH 8.0, with human neutrophil elastase (80 nM) (a) or trypsin (180 nM) (b) for 5 min at room temperature. Residual enzyme activity was then assayed with appropriate substrate.

(Figure 2). The inhibitor sample saturated with trypsin (excess of enzyme over inhibitor) still possessed full elastase-inhibitory capacity (Figure 2a). On the other hand, when an excess of elastase was present there was no trypsin inhibition (Figure 2b). Significantly, when elastase-inhibitory activity was only partially saturated, the sample preserved trypsin-inhibitory capacity. The result from titration assays was further confirmed in SDS/PAGE of inhibitor/proteinase reaction mixtures. As expected for serpins, the SDS-stable complexes of M_r about 80 000 were formed with either trypsin or elastase, but only when the inhibitor was first made to react with trypsin and then with elastase, the protein band of apparently native inhibitor (M_r 58 000) totally disappearing (results not shown). These data indicated that two inhibitors were present in the purified material, probably representing equimolar amounts of a trypsin inhibitor and an elastase inhibitor, with the former being sensitive to elastase inactivation. This assumption was further supported by N-terminal sequence analysis, when a mixed sequence was noted, as follows: LEU-PRO-GLU-ASN-VAL-Thr/Val-Pro/Val-Glu/Lys-ASP-GLN-His/Asp-Lys/Arg-Gly/Arg-Thr/Ala-Ala/His-VAL-ASP-ASP-HIS-Ala/Thr-LEU-ALA-SER-SER-ASN, where amino acids typed in capitals indicate that a single residue was identified at a given Edman degradation cycle, whereas amino acid codes divided by a slash and typed in lower case indicate that equimolar amounts of these two residues were detected at a given cycle. This clearly indicated the presence in the sample of two closely related proteins with 25 identical residues within the first 35 positions (Table 1).

Because conventional chromatography methods had failed to separate elastase and trypsin inhibitors, the purification of single forms of each goat plasma inhibitor was accomplished by negative adsorption of one of the proteins on trypsin–Sephacel or chymotrypsin–Sephacel columns. Unretarded protein which passed through the chymotrypsin–Sephacel column had exclusively trypsin-inhibitory activity, and after chromatography on Mono Q f.p.l.c. was eluted as a single peak. In SDS/PAGE it migrated as a single band of M_r 58 000 (Figure 1, lane h) accompanied by a dimer. The N-terminal sequence analysis of the first 40 residues is shown in Table 1.

The trypsin–Sephacel column flow-through of the inhibitor mixture (Figure 1, lane c) was resolved into two peaks by ion-

Table 1 Comparison of the amino-terminal sequence of goat trypsin inhibitor (contrapsin) and elastase inhibitor (elastasin) with their counterparts from human (α_1 -ACHY), sheep and bovine plasma

Residues in parentheses are the missing part of the N-terminal sequence, due to proteolytic cleavage during purification on the trypsin–Sepharose column.

Residue no. ...	1	5	10	15	20	25	30	35
Mixture of inhibitors	L P E N V	T P E V V K	D Q H K G T A	V D D H A	L A S S N T D F A F	L R	L Y K Q	
Goat contrapsin	L P E N V V V K D Q D R R A H V D D H T L A S S N T D F A F R L Y K N L A L K							
Goat elastasin	(L P E N V T P E D Q H K) G T A V D D H A L A S S N T D F A F L L Y K Q							
Human α_1 -ACHY	N S P L D E E N L T Q E N Q D R G T H V D - L G L A S A N V D F A F S L Y K Q L V L K							
Bovine contrapsin*	L P E N V V V K D Q T R R - - V D G H H L A S S N T D F A F S L Y							
Sheep contrapsin†	L P E N V V V K D Q D R R A S V D D L							

* J. Potempa (unpublished work).

† Sequence according to Sinha et al. (1988).

Table 2 Amino acid sequence of goat contrapsin–pig trypsin (a) and goat elastasin–neutrophil elastase (b) complexes

(a) Pig trypsin (410 pmol) (T), or trypsin previously inactivated by treatment with di-isopropyl fluorophosphate (T*), was incubated with goat contrapsin (420 pmol) in NH_4HCO_3 buffer, pH 7.8. After 60 s the sample was applied directly to the sequenator for six cycles. Proteolytically modified contrapsin was obtained by chromatography of the inhibitor on a trypsin–Sepharose column.

(b) Human neutrophil elastase (330 pmol) (E) or di-isopropyl fluorophosphate-treated elastase (E*) was incubated with 350 pmol of elastasin in NH_4HCO_3 buffer, pH 7.8, and after 60 s the sample was directly applied to the sequenator. I, inhibitor.

Residue identified							
(a) Trypsin inhibitor (contrapsin)					(b) Elastase inhibitor (elastasin)		
Cycle	I + T*	Inhibitor–trypsin complex	Modified inhibitor	Difference	I + E*	Inhibitor–elastase complex	Difference
1	Leu, Ile	Leu, Ile, Ala, Thr	Ala, Thr	Thr	Gly, Ile	Gly, Ile, Ser	Ser
2	Pro, Val	Pro, Val, His	His, Val	Val	Thr, Val	Thr, Val, Ser	Ser
3	Glu, Gly	Glu, Gly, Val, Leu	Val, Leu	Leu	Ala, Gly	Ala, Gly, Leu	Leu
4	Asn, Gly	Asn, Gly, Asp, Arg	Asp, Arg	Arg	Val, Gly	Val, Gly, Thr	Thr
5	Val, Tyr	Val, Tyr, Asp, Thr	Asp, Thr	Thr	Asp, Arg	Asp, Arg, Thr	Thr
6	Val, Thr	Val, His, Thr	His, Thr	Thr	Asp, Arg	Asp, Arg, Ile	Ile
7					His, Ala	His, Ala, Val	Val
8					Ala, Arg	Ala, Arg	Arg
9					Leu, Pro	Leu, Pro, Phe	Phe
10					Ala, His	Ala, His, Asn	Asn

exchange chromatography on Mono Q f.p.l.c. The first peak having elastase- and chymotrypsin-inhibitory activity was eluted at 0.2 M NaCl. As for the trypsin inhibitor, the active elastase inhibitor migrates in SDS/PAGE as a band of M_r 58000, accompanied by a dimer and a minute amount of partially degraded trypsin inhibitor of lower M_r (Figure 1, lane d). The minor bands visible between monomer and dimer are probably a result of dimerization of the partially degraded inhibitor. The second inactive protein peak was eluted from the Mono Q column immediately after the elastase inhibitor at 0.25 M NaCl. N-terminal sequence analysis of the protein in this peak indicated it as a proteolytically modified trypsin inhibitor (Table 2a). Significantly, on SDS/PAGE it migrated as two bands, but no protein dimer was observed (Figure 1, lane e). N-terminal sequence analysis of the elastase inhibitor reveals that the protein sequence begins at residue 13 (Table 1). It apparently has lost the first 12 amino acid residues, due to cleavage of a Lys–Gly peptide bond during chromatography on trypsin–Sepharose. Such an N-

terminally truncated elastase inhibitor was used in kinetic studies, but it is rather unlikely that it interacts with proteinases any differently from full-length inhibitor. Many inhibitory serpins do not have any extension in front of the first defined secondary structure, helix A, which in human α_1 -ACHY begins at residue 20 (Baumann et al., 1991). A comparison of the double sequence at the N-terminal of the inhibitor mixture with the N-terminus of the separated trypsin and elastase inhibitors enables the deduction of a consensus N-terminal sequence for each protein (Table 1).

Peptide-bond cleavage in trypsin–contrapsin and elastase–elastasin complexes

When the trypsin–contrapsin complex was submitted to N-terminal sequence analysis, four residues were obtained in each cycle (Table 2a). These were due to pig pancreatic trypsin, native inhibitor, proteolysis by trypsin of an Arg–Ala peptide bond

Each serpin (15 μ M) was incubated with proteinase at an inhibitor:enzyme molar ratio in the range from 1:1 to 1:10 000 at 37 °C. At various time intervals, samples were withdrawn and assayed for inhibitory activity against the cognate proteinase. For each reaction the time (in min) necessary for 50% inhibitor inactivation was divided by a value of the inhibitor:enzyme ratio, and the reciprocal was assumed to be the relative rate of proteolytic inactivation of the inhibitor. The values in the Table are the averages of 3–4 experiments performed at different inhibitor:enzyme ratios; n.d., not determined.

	α_1 -ACHY	Elastasin	Contrapsin
<i>P. aeruginosa</i> elastase	125.00	0.67	0.56
<i>S. marcescens</i> 56 kDa metalloproteinase	100.00	0.05	0.07
<i>Staph. aureus</i> metalloproteinase	16.95	0.12	< 0.01*
Human pancreatic trypsin (cationic)	58.82	< 0.01*	— †
Pig trypsin	3.33	< 0.01*	— †
Human neutrophil elastase	5.00	— ‡	0.50
Clostripain	n.d.	< 0.01*	66.70

‡ Elastasin is elastase inhibitor.

Sequencing of the human neutrophil elastase-elastasin complex revealed three residues in each cycle, one corresponding to neutrophil elastase, the second to active inhibitor, and the third due to proteolysis by elastase at the reactive site of the inhibitor (Table 2b). Sequence analysis of the equimolar mixture of diisopropyl fluorophosphate-treated proteinase and elastase inhibitor resulted only in the first two sequences.

N-terminal sequence analysis of proteolytically modified inhibitors yielded two residues in each cycle. For elastasin, one sequence was easily recognized as the N-terminal sequence of the truncated form of inhibitor obtained after chromatography on trypsin-Sepharose, and the second as the sequence within the reactive-site loop. The N-terminal sequence of contrapsin was only found in samples digested with clostripain or *P. aeruginosa* elastase. Incubation of this inhibitor with human elastase, *Staph. aureus* metalloproteinase, *S. marcescens* 56 kDa metalloproteinase and thermolysin caused not only the inactivation cleavage within the reactive-site loop but also cleavage at the N-terminal region of the protein. All cleavage sites are summarized in Table 4. The overlapping sequences generated from these data and from analysis of inhibitory complexes indicate Leu-Ser and

Key: *reactive-site peptide bond; 1, thermolysin; 2, *Staph. aureus* metalloproteinase, 3, *S. marcescens* 56 kDa metalloproteinase; 4, *P. aeruginosa* elastase; 5, human neutrophil elastase; 6, clostripain; 7, bovine and human trypsin; 8, human neutrophil elastase. The amino acid residues identical in human α_1 -ACHY and the goat inhibitors are capitalized, and the gap was introduced to facilitate sequence alignment. Cleavage sites of α_1 -ACHY are as given by Potempa et al. (1991).

N-terminal sequence of contrapsin:
Leu Pro Glu Asn Val Val Val Lys Asp Gln Asp Arg Arg Ala His
 2 5 1,3

Reactive-site loop of contrapsin:
Xaa P₁ P₁'
(Arg)* Thr Val LEU --- --- --- ARG THR Thr VAL ARG PHE Asp ARG PRO PHE LEU Ile Ala
6 1-5

Reactive-site loop of elastasin :
Xaa LEU * SER Ser LEU --- --- --- Thr THR ILE VAL ARG PHE ASN ARG PRO PHE LEU Ile Leu
2 1,3,4

Reactive-site loop of α₁-ACHY:
Val Lys Ile Thr Leu LEU * SER Ala LEU Val Glu Thr ARG THR ILE VAL ARG PHE ASN ARG PRO PHE LEU Met Ile
7 8 3 2,4

Table 5 Second-order association rate constants of various proteinases with goat contrapsin and elastasin

k_{ass} was considered 0 if after 120 min preincubation of molar excess of inhibitor with proteinase there was no enzyme inhibition. Abbreviation: n.d., not determined.

Proteinase	k_{ass} ($\text{M}^{-1} \cdot \text{s}^{-1}$)	Elastasin	
		Native	Oxidized
Human plasmin	2.3×10^3	0	n.d.
Pig trypsin	1.9×10^6	0	n.d.
Human neutrophil elastase	0	1.5×10^6	1.0×10^6
Pig pancreatic elastase	0	2.5×10^4	2.2×10^4
Cathepsin G	n.d.	6.7×10^4	6.8×10^4
α -Chymotrypsin	0	1.5×10^5	1.5×10^5
Human α -thrombin	0	0	0

Xaa-Thr as the $\text{P}_1\text{-P}_1'$ residues of the inhibitory reactive sites of elastasin and contrapsin, respectively. Although the P_1 amino acid residue of goat contrapsin was not determined experimentally, it can be inferred indirectly that it is an arginine residue, because the inhibitor is very sensitive to proteolytic inactivation, due to cleavage of its putative $\text{P}_1\text{-P}_1'$ peptide bond by clostripain and gingipain, which are cysteine proteinases with strict arginine-specificity (Gilles et al., 1979; Chen et al., 1992). In addition, Arg at the P_1 site is in keeping with the narrow inhibitory specificity of this serpin.

Association rate constants of native and oxidized inhibitors

Second-order association rate constants (k_{ass}) for the inhibition of different proteinases by each goat inhibitor are summarized in Table 5. The specificity of each inhibitor is in agreement with the amino acid residue occupying the P_1 position. In addition, we have also examined the effect of elastasin on partially purified elastases from bovine, horse and pig neutrophil granules. In all cases the association rate constant was estimated to be near $10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ (results not shown), suggesting that goat neutrophil elastase would also be efficiently inhibited. Significantly, cleavage of the first 12 amino acid residues of elastasin has no effect on inhibitor interaction with proteinases. Furthermore, preincubation of either inhibitor with a 30-fold molar excess of *N*-chlorosuccinimide led to oxidation of only one of the six methionine residues in either protein (results not shown), but had no effect on proteinase inhibition (Table 5).

DISCUSSION

A large number of proteins belonging to the Serpin superfamily have been isolated and characterized from human plasma, tissues and cell lines (Marshall, 1993), including several which have recently been described (Coughlin et al., 1993; Chai et al., 1993; Steele et al., 1993). Nevertheless, only a few investigative efforts have been made to characterize structurally related serpins from other species. This is particularly true for α_1 -ACHY, the second major serine proteinase inhibitor present in human plasma (based on its plasma concentration). Recently, however, an anti-chymotrypsin-like inhibitor has been purified from the bovine pituitary gland, which was identified as a regulator of a cysteine proteinase involved in enkephalin precursor processing (Hook et al., 1993). It was also shown that inhibitors immunologically cross-reactive with antiserum to human anti-chymotrypsin are

present in goat plasma (Vankan and Bell, 1993); however, no such protein has actually been purified from plasma of animals other than rodents.

In this study, we have isolated from goat plasma two related inhibitors whose N-terminal sequence demonstrated that they are proteins homologous to human α_1 -ACHY, although their inhibitory spectra were in direct contrast. One of the proteins was found to be the exclusive inhibitor of trypsin, and is therefore referred to as contrapsin, although its N-terminus shares little identity with that of the rat (Ohkubo et al., 1991a), mouse (Ohkubo et al., 1991b) or guinea-pig (Suzuki et al., 1991) trypsin inhibitors. The difference is even more significant within the reactive-site loop, where goat contrapsin shows higher similarity to human anti-chymotrypsin (Table 4) than to any of the rodent inhibitors.

Previously, two independent laboratories used very different methods of isolation in the separation of goat α_1 -PI (Hercz, 1982; Salahuddin, 1991), a potent inhibitor of bovine trypsin. However, the protein(s) remained grossly uncharacterized with respect to both structure and kinetics of interaction with proteinases. This lack of information made difficult any comparison between purified goat α_1 -PI and contrapsin, although it is likely that they represent the same protein as that described here. An inhibitor with properties very similar to those of goat contrapsin has also been purified from sheep plasma and categorized as α_1 -PI (Sinha et al., 1988). However, comparison of the N-terminal sequence revealed that the sheep and goat proteins were almost identical (Table 1), indicating that the sheep inhibitor should be re-classified as a contrapsin and implying that occurrence of this type of serpin is not limited to the rodents, but is widespread among at least one order (artiodactyl) or more of mammals. Indeed, sheep orthodox α_1 -PI has recently been isolated and thoroughly characterized (Mistry et al., 1991), and its amino acid sequence has been deduced from cDNA sequence analysis (Brown et al., 1989).

The second inhibitor purified from goat plasma seems to have a more definite target, neutrophil elastase, and is therefore referred to as elastasin. It is also a reasonably good inhibitor of α -chymotrypsin, cathepsin G and pancreatic elastase, this broad inhibitory spectrum being in line with the P_1 Leu residue. A recombinant form of human α_1 -PI with Met substituted for the P_1 Leu exhibited very similar inhibitory spectrum (Jallat et al., 1986) to elastasin, and this confirms the general belief that the P_1 amino acid of serpins primarily dictates the proteinase specificity of an inhibitor. Elastasin has another feature of the P_1 Ala, Ile, Leu or Val recombinant forms of α_1 -PI (Travis et al., 1985; Matheson et al., 1986; Jallat et al., 1986), namely its resistance to oxidative inactivation. Because more than 90% of the goat plasma elastase-inhibitory capacity is also oxidation-resistant (Potempa et al., 1990) and correlates with the calculated plasma concentration of elastasin, it is apparent that this serpin constitutes the major anti-elastase of goat blood. Therefore, although no inhibitory measurements have been done with goat proteinases, we hypothesize that elastasin is the inhibitor that controls neutrophil elastase activity *in vivo*, fulfilling in this way the function of α_1 -PI from other species.

Although both goat elastasin and contrapsin share a high degree of sequence identity with human anti-chymotrypsin as a part of the reactive-site loop downstream from the $\text{P}_1\text{-P}_1'$ bond, they differ considerably from the human inhibitor in their susceptibility to proteolytic inactivation. Except for contrapsin inactivation by clostripain, both goat inhibitors are very slowly cleaved by all proteinases investigated. In fact, in comparison with human anti-chymotrypsin, they can be considered resistant to proteolytic inactivation. It is puzzling why anti-chymotrypsin is

inactivated at a rate more than 1000-fold faster than for the goat inhibitors, even though in some cases inactivation occurs through cleavage of very similar peptide bonds, e.g. Ala-Leu in α_1 -ACHY, compared with Ser-Leu or Val-Leu in elastasin and contrapsin respectively (Table 4). The answer to this question probably rests in the conformation of the reactive-site loop. In the goat inhibitors the loop is three residues shorter than that in anti-chymotrypsin, but this may not explain the phenomenon, since some other serpins (e.g. α_1 -PI, C1-inhibitor, α_2 -antiplasmin) have even shorter loops, but are still vulnerable to proteolytic inactivation (Moriwaka et al., 1984; Kress, 1986; Potempa et al., 1986; Mast et al., 1991b). Presumably, some other factors are involved in determining susceptibility to proteolytic cleavage, and it is likely that only a comparison of the crystal structure of the native forms of these serpins can resolve this question.

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