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Comparative properties of three functionally different but structurally related serpin variants from horse plasma

Jan POTEMPA,* John K. WUNDERLICH† and James TRAVIS‡§

*Institute of Molecular Biology, Jagiellonian University, 31-120 Cracow, Poland, †Department of Chemistry, University of Georgia, Athens, GA 30602, U.S.A., and ‡Department of Biochemistry, University of Georgia, Athens, GA 30602, U.S.A.

Three structurally related but functionally different serpins from horse plasma were isolated and characterized. In spite of their identical *N*-terminal sequences, which show some similarity to that of human α_1-proteinase inhibitor, the reactive-centre loops of each of these proteins show extensive variation. Only inhibitor I, with a P_1 methionine residue, resembles human α_1-PI with regard to (*a*) similarity of amino acid sequence in the vicinity of the reactive-site peptide bond, (*b*) broad inhibitory specificity, (*c*) sensitivity to oxidative inactivation and (*d*) high rate of reactivity with neutrophil elastase(s). Inhibitor II, with a P_1 arginine residue, is an exclusive trypsin inhibitor, and inhibitor III is an oxidation-resistant slow-reacting elastase inhibitor with a P_1 alanine residue. Comparison of association rate constants for the inhibition of horse neutrophil elastases by the three inhibitors indicates that only inhibitor I is likely to be physiologically important in the regulation of these enzymes.

INTRODUCTION

Human α_1-proteinase inhibitor (α_1-PI) is a plasma glycoprotein which is responsible for more than 90% of the trypsin- and elastase-inhibitory capacity in this fluid [1]. The pathophysiological importance of this inhibitor arises from the fact that an inborn deficiency in its secretion (ZZ phenotype) can lead to the development of familial emphysema [2]. The aetiology of this disease is well established and results from an imbalance between available α_1-PI and leucocyte elastase in the lower respiratory tract [3], favouring uncontrolled proteolysis by the latter. It is also believed that inactivation, by oxidation of the P_1 methionine residue of α_1-PI, can result in a functional imbalance between inhibitor and elastase in normal individuals. Indeed, the strong correlation between inhalation of cigarette smoke and the risk of emphysema is almost certainly due to oxidative inactivation of α_1-PI by components produced either directly from the smoke or by neutrophils attracted to the lung [4–7].

Besides man, the only other animal species known to suffer from spontaneous chronic lung disease is the horse [8–10]. This animal has a high level of elastase-inhibitory activity in both its plasma and the cytosol of its neutrophils, the latter having been found to possess an oxidation-resistant elastase inhibitor [11,12]. In contrast, the sheep, which has often been used as an animal model of human lung disease, has low plasma antielastase activity and low elastase activity in its neutrophils. We have recently demonstrated that nearly 60% of horse plasma elastase-inhibitory activity is also oxidation-resistant [13], suggesting that more than one type of inhibitor is present in this fluid. Functional heterogeneity of multiple forms of horse α_1-PI has been previously reported [14,15], together with the suggestion that the deficiency of a specific elastase isoinhibitor could be responsible for the development of lung disease in the horse [14]. In the present paper we describe the isolation of three different functional inhibitors from horse plasma, two of which can inactivate horse neutrophil elastase(s), together with reactive-site loop amino acid sequences and kinetics of the interaction of each with different proteinases.

MATERIALS AND METHODS

Materials

Horse leucocyte elastases 2A and 2B and human neutrophil elastase were purified according to previously published procedures [16,17]. Human thrombin was a gift from Dr. J. Fenton (New York State Health Department, Albany, NY, U.S.A.). All other enzymes and chemicals of at least analytical grade were from Sigma Chemical Co.

Purification of horse plasma inhibitors

The purification scheme for the isolation of horse plasma inhibitors was based, initially, on the procedure described by Pannel *et al.* [18] for the isolation of human α_1-PI. It consisted of the following steps: (1) $(NH_4)_2SO_4$ precipitation; (2) Cibacron Blue–Sepharose chromatography; (3) DEAE-cellulose (DE-52) ion-exchange chromatography at pH 6.8 and rechromatography at pH 8.0; (4) gel filtration on Sephadex G-100. Homogeneity of the inhibitor was checked by both SDS/PAGE and crossed immunoelectrophoresis against rabbit antiserum to whole horse plasma.

The separation of different isoinhibitors was obtained by ion-exchange chromatography in the Pharmacia f.p.l.c. system with a Mono Q column. To distinguish between different forms of horse inhibitors, the inhibitory capacity for trypsin and human leucocyte elastase was determined in each fraction, both before and after *N*-chlorosuccinimide treatment, and inhibitory activity was related to protein content. Only the fractions considerably enriched in a single form of inhibitor were collected, and these were rechromatographed until single forms of each inhibitor were obtained.

The three proteinase inhibitors from horse plasma were separated from each other as follows: the oxidation-sensitive elastase inhibitor (inhibitor I) was obtained by chromatography of the mixture obtained after step 4, above, on a Mono Q column equilibrated with 20 mM-Tris/HCl buffer, pH 8.0, with a shallow gradient of 0.1–0.3 M-NaCl. Protein fractions not containing this inhibitor were collected, dialysed against 20 mM-sodium phos-

Abbreviation used: α_1-PI, α_1-proteinase inhibitor.

§ To whom correspondence should be addressed.

Table 1. Comparison of *N*-terminal sequence of horse inhibitors with human α_1-PI

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
Horse inhibitors I, II, III:*	Glu-	Asp	-Leu-	Gln-	Gly-	Asp-	Ala	-Val-	Pro-	Glu-	Arg-	His-	Ala-	Thr-	Lys-	Asp	Asp	-Asn-	Glu-	His-	Pro	-Gln-	Glu-	Pro-	Ala-	Glu-	His-	Lys-	Lys-	Ala-	Pro-	Asn
Human α_1-PI:	Glu-	Asp	-Pro-	Gln-	Gly-	Asp-	Ala	-Ala-	Gln-	Lys-	Thr-	Asp-	Thr-	Ser-	His-	His	Asp	-Gln-	Asp-	His-	Pro	-Thr-	Phe-	Asn-	Lys-	Ile-	Thr-	Pro-	Asn-	Leu-	Ala-	Glu

* All horse inhibitors had identical *N*-terminal sequences through residues 1–32.

phate buffer, pH 6.5, and applied to a Mono Q column equilibrated with the same buffer. Separation of the trypsin inhibitor (inhibitor II) from the oxidation-resistant elastase inhibitor (inhibitor III) was achieved with a gradient of 0.05–0.2 M-NaCl.

Active-site titration

All enzymes utilized in kinetic studies were active-site-titrated either with burst titrants (trypsin and thrombin) [19] or by titration against a secondary standard such as human α_1-PI (human leucocyte elastase and horse leucocyte proteinases 2A and 2B). Active-site titration of α_1-PI was made against a standardized solution of pig trypsin.

Sequence analysis

Terminal sequence analysis of the *Staphylococcus aureus*-V8-proteinase-inactivated horse inhibitors or proteinase–horse proteinase inhibitor complexes was performed with an Applied Biosystems 4760A gas-phase Sequenator, using the program designed by the manufacturer. Analysis of complexes was made by first incubating either of the elastase inhibitors (520 pmol) and human leucocyte elastase (480 pmol), or the trypsin inhibitor (580 pmol) and pig trypsin (490 pmol) for 60 s at room temperature in 0.02 M-ammonium bicarbonate buffer, pH 8.0. The samples were then transferred directly to the Sequenator and dried under vacuum before initiation of the program. Controls containing di-isopropyl phosphorofluoridate-treated proteinases, native horse inhibitors or equimolar mixtures of each were also subjected to sequence analysis.

Limited proteolysis of horse inhibitors

Individual horse inhibitors were inactivated by limited proteolysis with bacterial proteinases. Each inhibitor (50–100 nmol) was incubated at an inhibitor/enzyme molar ratio between 5:1 and 1000:1 in 0.1 M-Tris/HCl buffer, pH 8.0 (*S. aureus* V8 proteinase), or in the same buffer supplemented with 0.5 mM-$CaCl_2$ and 1.75 mM-dithiothreitol (clostripain). At given time intervals, samples were removed and tested for residual inhibitory activity towards either pig trypsin or human neutrophil elastase. The effectiveness of inhibitor cleavage was confirmed by SDS/PAGE [20] with an 8–20% (w/v) acrylamide linear gradient. When inhibitor activity was decreased to near 10% of controls the reaction was stopped by separation of the modified inhibitor from proteinase by using Mono Q f.p.l.c., as described above. Fractions containing inhibitor were freeze-dried, redissolved in water, desalted by passing through a Sephadex G-25 column (0.8 cm × 5.0 cm) and subjected to sequence analysis.

Measurement of association rates

The second-order association rate constants for native and oxidized horse α_1-PI isoforms with various mammalian serine proteinases were determined by the method of Bieth [21]. Equimolar mixtures of enzyme and inhibitor (based on the activities of each protein) were incubated for increasing time periods at room temperature in a total volume of 1.0 ml of 0.2 M-Tris/HCl buffer, pH 8.0. Residual enzyme activity was then measured by the addition of saturating amounts of specific proteinase substrates.

RESULTS

Purification of horse inhibitors

The scheme utilized for the isolation of a mixture of horse α_1-PI-related inhibitors, following the procedure for human α_1-PI, resulted in a yield of about 135 mg of protein from 500 ml of plasma. The content of the three different isoinhibitors in the mixture was estimated by titration of both native or oxidized

Table 2. Amino acid sequence of horse plasma inhibitor–target proteinase complexes

Abbreviations: I, inhibitor; T and T* or E and E*, active and di-isopropyl phosphorofluoridate-inactivated trypsin or leucocyte elastase respectively.

	Residues identified								
	Trypsin inhibitor (inhibitor II)			Oxidation-sensitive inhibitor (inhibitor I)			Oxidation-resistant inhibitor (inhibitor III)		
Cycle	I+T*	I–T complex	Difference	I+E*	I–E complex	Difference	I+E*	I–E complex	Difference
1	Glu, Ile	Glu, Ile, Thr	Thr	Glu, Ile	Glu, Ile, Ser	Ser	Glu, Ile	Glu, Ile, Thr	Thr
2	Asp, Val	Asp, Val, Leu	Leu	Asp, Val	Asp, Val, Leu	Leu	Asp, Val	Asp, Val, Leu	Leu
3	Pro, Gly	Pro, Gly, Leu	Leu	Leu, Gly	Leu, Gly, Pro	Pro	Leu, Gly	Leu, Gly, Leu	Leu
4	Gln, Gly	Gln, Gly, His	His	Gln, Gly	Gln, Gly, Pro	Pro	Gln, Gly	Gln, Gly, Leu	Leu
5	Gly, Tyr	Gly, Tyr, Thr	Thr	Gly, Arg	Gly, Arg, Glu	Glu	Gly, Arg	Gly, Arg, Asp	Asp
6	Asp, Thr	Asp, Thr, Asn	Asn	Asp, Arg	Asp, Arg, Leu	Leu	Asp, Arg	Asp, Arg, Asn	Asn
7				Ala	Ala, Glu	Glu	Ala	Ala, Val	Val
8				Val, Arg	Val, Arg, Phe	Phe	Val, Arg	Val, Arg, Glu	Glu
9				Pro	Pro, Asn	Asn	Pro	Pro, Phe	Phe
10				Glu, His	Glu, His, Arg	Arg	Glu, His	Glu, His, Asn	Asn

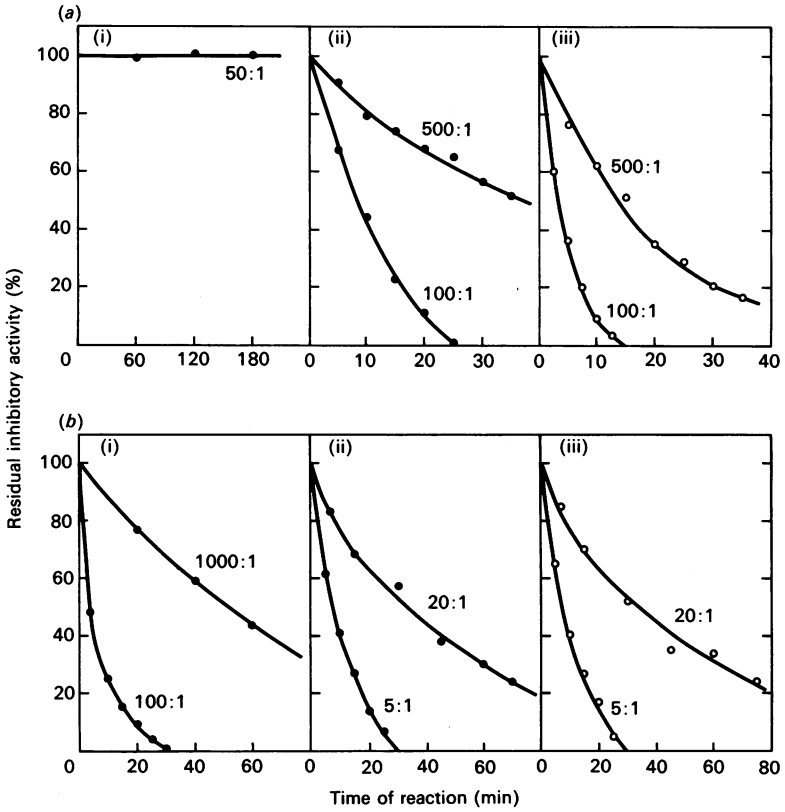

Fig. 1. Time- and concentration-dependent inactivation of horse plasma inhibitors by bacterial proteinases

Various molar ratios of inhibitor to enzyme were incubated as described in the text, with samples being removed at given time periods and tested for either residual neutrophil-elastase-inhibitory activity (●) or trypsin-inhibitory activity (○). (i) Oxidation-sensitive elastase inhibitor (inhibitor I); (ii) oxidation-resistant elastase inhibitor (inhibitor III); (iii) trypsin inhibitor (inhibitor II). (*a*) Clostripain incubation; (*b*) *S. aureus* V8 proteinase incubation.

samples with pig pancreatic elastase, trypsin and human neutrophil elastase. This estimation was possible because of the different enzyme specificities of each inhibitor form as well as their differing sensitivity to oxidative inactivation. It was found that the mixture contained about 28% of inhibitor I (inhibits neutrophil elastase and trypsin but not pancreatic elastase), 49% of inhibitor III (inhibits both elastases but not trypsin) and 34% of inhibitor II (exclusive trypsin inhibitor).

Separation of inhibitor I was achieved by applying the mixture to a Mono Q column f.p.l.c. system equilibrated with 20 mM-Tris/HCl buffer, pH 8.0. The inhibitor was eluted by a linear NaCl gradient at 0.19 M-NaCl in comparison with the other iso-inhibitors, which were eluted between 0.21 M- and 0.24 M-NaCl.

The isolation of inhibitors II and III was accomplished by chromatography on a Mono Q column f.p.l.c. system equilibrated with 20 mM-sodium phosphate buffer, pH 6.5. Inhibitor II was

eluted first at a salt concentration of 0.1 M followed by inhibitor III at 0.13 M-NaCl. Titration experiments indicated that the final three purified inhibitors were approx. 90% active. In SDS/PAGE (results not shown) inhibitor I migrated as two bands of M_r 52000 and 53000 whereas inhibitors II and III gave single bands of M_r 56000 and 54000 respectively.

N-Terminal sequence analysis through 32 residues of each horse inhibitor gave only a single sequence for all three proteins (Table 1). This suggests that these proteins are very closely related to each other and that their individual specificities must be due to structural differences in other areas, but especially in the reactive-site loop.

Reactive-site structure

When trypsin in complex with its inhibitor (inhibitor II) was submitted to sequencing, three residues were obtained in each cycle (Table 2). Two of these were due to trypsin and native inhibitor. The third sequence was as follows:

Thr-Leu-Leu-His-Thr-Asn
(inhibitor II)

These data indicate that complex-formation with trypsin occurred by attack of the enzyme at an Xaa–Thr reactive-site peptide bond. Similar results were obtained when the elastase–elastase inhibitor complexes were sequenced. Besides the *N*-terminal sequences of enzyme and native inhibitor, the new sequences were determined to be as follows:

Ser-Leu-Pro-Pro-Glu-Leu-Glu-Phe-Asn-Arg-Pro-Phe
(inhibitor I)

Thr-Leu-Leu-Leu-Asp-Asn-Val-Glu-Phe-Asn-Arg-Pro-Phe
(inhibitor III)

These data suggest cleavage at Xaa–Ser and Xaa–Thr reactive sites for inhibitors I and III respectively.

Incubation of inhibitor I, II or III with catalytic amounts of *S. aureus* V8 proteinase and inhibitor II or III with clostripain resulted in rapid loss of inhibitory activity (Figs. 1*b* and 1*a* respectively), presumably because of limited proteolysis at or near the reactive site. Significantly, there was a major difference in this sensitivity for proteolytic inactivation among the three inhibitors. Specifically, inhibitor I was completely resistant to inactivation by clostripain (Fig. 1*a*) but was most sensitive for proteolytic cleavage by the V8 proteinase (Fig. 1*b*). This indicates that considerable structural differences around the reactive site of the horse inhibitor must exist in spite of identical *N*-terminal sequences.

When V8-proteinase-treated inhibitors were directly submitted to *N*-terminal sequence analysis, three residues were obtained in each step up to ten cycles, because of cleavage in the reactive-site loop as well as between residues 10 and 11 (Glu–Ala) of the native proteins. However, sequencing of f.p.l.c.-purified modified forms of inhibitors gave only two parallel sequences (Table 3), the *N*-terminal decapeptide having been separated. These data enabled us to determine the reactive sites of all three inhibitors by overlap with those described above (Table 2). These are as follows:

Inhibitor I (oxidation-sensitive elastase inhibitor):

(Glu)-Met-Ile-Pro-MET-SER-Leu-Pro-Pro-Glu-Leu-Glu-Phe-
Asn-Arg-Pro-Phe-Ile-Leu-Ile-Ile-Tyr-Asp-

Inhibitor II (trypsin inhibitor):

(Glu)-Ala-Ile-ARG-THR-Leu-Leu-His-Thr-Asn-Val-Glu-Phe-
Asn-Arg-Pro-Phe-Val-Leu-Ile-Ile-Tyr-Asp-

Table 3. Amino acid sequence of *S. aureus*-V8-proteinase-inactivated horse inhibitors

Modified inhibitors were purified in a Mono Q column f.p.l.c. system before being sequenced, and their *N*-terminal sequence begins at residue 11 (D) because of Glu–Arg peptide-bond cleavage by the V8 proteinase.

	Residues identified			
Cycle	A (inhibitor II)	B (inhibitor I)	C (inhibitor III)	D *N*-terminal sequence (residues 11–33)
1	Arg, Ala	Arg, Met	Arg	Arg
2	His, Ile	His, Ile	His, Pro	His
3	Ala, Arg	Ala, Pro	Ala	Ala
4	Thr	Thr, Met	Thr	Thr
5	Lys, Leu	Lys, Ser	Lys, Leu	Lys
6	Asp, Leu	Asp, Leu	Asp, Leu	Asp
7	Asp, His	Asp, Pro	Asp, Leu	Asp
8	Asn, Thr	Asn, Pro	Asn, Asp	Asn
9	Glu, Asn	Glu	Glu, Asn	Glu
10	His, Val	His, Leu	His, Val	His
11	Pro, Glu	Pro, Glu	Pro, Glu	Pro
12	Gln, Phe	Gln, Phe	Gln, Phe	Gln
13	Glu, Asn	Glu, Asn	Glu, Asn	Glu
14	Pro, Arg	Pro, Arg	Pro, Arg	Pro
15	Ala, Pro	Ala, Pro	Ala, Pro	Ala
16	Glu, Phe	Glu, Phe	Glu, Phe	Glu
17	His, Val	His, Ile		His
18	Lys, Leu	Lys, Leu		Lys
19	Lys, Ile	Lys, Ile		Lys
20	Ala, Ile	Ala, Ile		Ala
21	Pro, Tyr	Pro, Tyr		Pro
22	Asn, Asp	Asn, Asp		Asn

Inhibitor III (oxidation-resistant elastase inhibitor):

(Glu)-Arg-Pro-ALA-THR-Leu-Leu-Leu-Asp-Asn-Val-Glu-Phe-
Asn-Arg-Pro-Phe-

Thus MET-SER, ARG-THR and ALA-THR represent the P_1–P_1' residues in the inhibitor reactive site of inhibitors I, II and III respectively. Owing to the limited specificity of the V8 proteinase [22], it is expected that in all cases inhibitor inactivation occurred by cleavage at a Glu–Xaa peptide bond. The lack of an arginine residue in the reactive-site region of the oxidation-sensitive elastase inhibitor most probably explains the resistance of this inhibitor to proteolytic inactivation by clostripain.

Association rate constants for native and oxidized inhibitors

Comparison of association rate constants revealed few significant conclusions. Only inhibitor I, which accounts for 40% of the elastase-inhibitory activity in plasma [14], was efficient against two major horse leucocyte elastases ($K_{ass.} > 10^7\ M^{-1} \cdot s^{-1}$). The remaining elastase-inhibitory activity is presumably due to inhibitor III, which had relatively low association rates with both of the two horse enzymes (Table 4). These weak association rate constants are somewhat surprising, since the P_1 position of the reactive site of inhibitor III is occupied by an alanine residue, which should be highly suitable for complex-formation with serine elastolytic enzymes. Inhibitor II, with a P_1 arginine, was, as expected, exclusively a trypsin inhibitor.

The oxidation of inhibitors in the presence of a 30-fold molar excess of *N*-chlorosuccinimide led to inactivation of only inhibitor I (Fig. 2). Unlike the oxidation of human α_1-PI, oxidative

Table 4. Second-order association rate constants of various proteinases with horse plasma inhibitors

Abbreviations: SucNCl, *N*-chlorosuccinimide; N.D., not determined.

	$K_{ass.}$ ($M^{-1} \cdot s^{-1}$)				
	Oxidation-sensitive elastase inhibitor (inhibitor I)		Oxidation-resistant elastase inhibitor (inhibitor III)		Trypsin inhibitor (inhibitor II)
Enzyme	Native	SucNCl-treated	Native	SucNCl-treated	
Porcine trypsin	1.2×10^5	0	0	N.D.	3.5×10^5
Pig pancreatic elastase	–*	N.D.	6.4×10^4	6.3×10^4	0
Human leucocyte elastase	6.0×10^7	0	2.0×10^6	1.9×10^6	0
Proteinase 2A	2.0×10^7	0	3.2×10^4	N.D.	0
Proteinase 2B	5.0×10^7	0	5.2×10^5	5.3×10^5	0
Human thrombin	0†	N.D.	0	N.D.	0

* Total pig pancreatic elastase inhibition requires more than a 10-fold molar excess of inhibitor.
† $K_{ass.}$ was considered to be 0 if after 120 min preincubation with a 2-fold molar excess of inhibitor there was no enzyme inhibition.

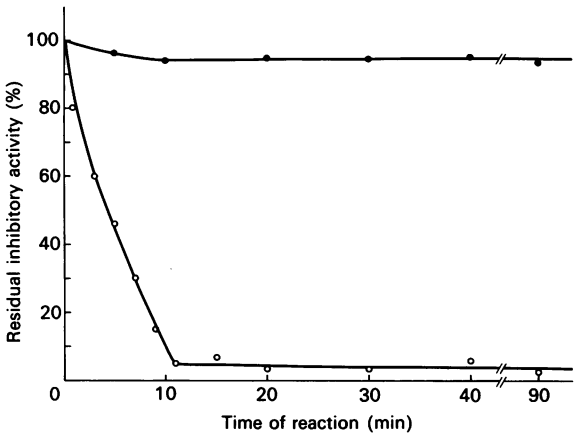

Fig. 2. Time-dependent oxidative inactivation of horse elastase inhibitors

Purified inhibitors (6.5 μM each) were incubated in the presence of *N*-chlorosuccinimide (200 μM) in 0.1 M-Tris/HCl buffer, pH 8.0, for various time periods and then assayed for residual neutrophil-elastase-inhibitory activity. ○, Inhibitor I; ●, inhibitor III.

modification of this inhibitor resulted in a loss of all inhibitory activity (Table 4), probably because of modification of the two methionine residues at the P_1 and P_4 positions.

DISCUSSION

Horse plasma α_1-PI has been purified and partially characterized in four independent laboratories [23–28]. Although it was obvious that such preparations contained at least two electrophoretically different but immunologically identical isoinhibitors [14,25,27], no real attempt was made to separate and characterize the different forms. Even greater heterogeneity of the horse proteinase-inhibitory system has been shown by a combination of polyacrylamide-gel isoelectric focusing and polyacrylamide-gel pore gradient electrophoresis [15,28–30]. From our preparation of horse α_1-PI we were able to separate and characterize three different inhibitors. These were found to differ from each other with regard to: (*a*) sensitivity to proteolytic cleavage by bacterial proteinases; (*b*) sensitivity to oxidative inactivation; (*c*) inhibitory specificity. On the other hand, all three inhibitors had an identical *N*-terminal sequence up to 32 residues, which suggested that they were also closely related to each other (Table 1).

Despite this remarkable identity at the *N*-terminus, horse α_1-PI isoforms differ significantly within the reactive-site loop (Table 5). In this region only inhibitor I demonstrates significant similarity to human α_1-PI. Indeed, eight of ten residues between the P_5 and P_5' subsites of the reactive site are identical. This inhibitor, after oxidation, loses all activity against elastases or trypsin (Table 4), whereas human α_1-PI is still active, slowly forming less stable inhibitory complexes with target proteinases [31,32]. This difference might be explained by oxidation of two methionine residues at the P_4 and P_1 positions in the horse inhibitor, in comparison with that of the P_8 and P_1 residues in human α_1-PI. A second difference between these two inhibitors is with regard to pancreatic elastase inhibition, the horse inhibitor being ineffective against this enzyme. Although it is clear that the P_1 residue dictates serpin specificity which is further modulated by adjacent residues, including those in P_1' positions [33], it may well be that, in the case of the horse inhibitor, specificity might

Table 5. Comparison of reactive-site sequences of horse serpins with human α_1-PI

	P_1–P_1'
Human α_1-PI:	Glu -Ala-Ile-Pro-MET-SER-Ile-Pro-Pro-Glu- – -Val-Lys-Phe-Asn-Lys-Pro-Phe-Val-Phe-Leu-Met-Ile
Inhibitor II (trypsin):	(Glu)-Ala-Ile- – -ARG-THR-Leu-Leu-His-Thr-Asn-Val-Glu-Phe-Asn-Arg-Pro-Phe-Val-Leu-Ile-Ile-Tyr
Inhibitor I (elastase):	(Glu)-Met-Ile-Pro-MET-SER-Leu-Pro-Pro-Glu- – -Leu-Glu-Phe-Asn-Arg-Pro-Phe-Ile-Leu-Ile-Ile-Tyr
Inhibitor III (elastase):	(Glu)-Arg- – -Pro-ALA-THR-Leu-Leu-Leu-Asp-Asn-Val-Glu-Phe-Asn-Arg-Pro-Phe

also involve the P_4 and P_2' subsites of the reactive centre, which are different for these two elastase inhibitors.

The reactive-site amino acid sequence of inhibitor II had, as was expected from its specificity, a P_1 arginine residue. A comparison of the amino acid sequence at the reactive centre of this inhibitor with human α_1-PI reveals some similarity, but major differences occur at or near the P_1 residue. Surprisingly, inhibitor II and human C1 inhibitor show significant sequence similarity in the vicinity of their reactive sites, including an Arg-Thr-Leu-Leu sequence between the P_1 and P_3' positions. The physiological function of horse inhibitor II is obscure at the moment, and it would be useful to study the interactions of this protein with the known target proteinases for C1 inhibitor.

Inhibitor III was determined to have a P_1 alanine residue, and this explains its resistance to oxidative inactivation [34]. Although this inhibitor forms complexes with pig pancreatic elastase, as well as with those from horse and human neutrophils, inhibition rates are rather low (Table 4). This is difficult to explain, since other inhibitors with a P_1 alanine residue [12,34,35] react with mammalian elastolytic enzymes very rapidly. Once more, this reiterates the importance of other residues in the vicinity of the reactive-site peptide bond, or elsewhere, in determining serpin specificity.

The pig [36,37], human [38,39], horse [40,41], mouse and rat [42] have multiple proteinase-inhibitor-related gene complexes. Indeed, three different contrapsin-related cDNA clones have been described in rodents [43,44], although nothing is known about their expression *in vivo*. In the horse, however, three forms of α_1-PI are expressed and present in plasma, and it is likely that genes encoding them were created by ancestral α_1-PI-related gene duplication followed by accelerated evolution of that part of the gene containing the reactive site, as was shown for the three rodent proteinase inhibitors [43]. It is postulated that mutations within the reactive-site loop are being rapidly fixed by positive Darwinian selection based on modification of inhibitory activity [43] or based on modified cleavage susceptibility [45]. It is thus likely that there will be great evolutionary pressure for the conservation of mutational changes that provide resistance to loop cleavage by pathogenic proteinases.

In the case of the horse, such guided evolution has created an oxidation-resistant elastase inhibitor (inhibitor III). Since oxidative inactivation of human α_1-PI by oxidants released by activated neutrophils is thought to be important in uncontrolled lung elastin degradation, ultimately leading to the development of emphysema [46–48], having inhibitor III would appear to be a distinct advantage for the horse. However, a close look at the association rate constants (Table 4) and calculation of a delay time [21] for elastase inhibition in horse plasma (1.0 s for horse elastase 2B, 15.5 s for horse elastase 2A) indicates that inhibitor III would be unlikely to protect lung elastin against degradation by horse leucocyte proteinases. In addition, the horse lung tissue is in permanent contact with many different proteolytic enzymes of bacterial and fungal origin, inhaled during feeding [49,50], which may proteolytically inactivate horse inhibitors. In this sense the presence of a P_3 arginine in inhibitor III makes it extremely sensitive to proteolytic inactivation by proteinases of trypsin-like activity, which are abundant in many of these organisms. Thus it is unclear as to why the equine system has developed this oxidation-resistant elastase inhibitor.

The only inhibitor that can efficiently control horse leucocyte elastases [51] is inhibitor I, which is susceptible to oxidative and proteolytic inactivation. However, plasma concentrations of this inhibitor are only about 25 % of that of human α_1-PI and could be considered as a mild inhibitor-deficiency state. Such low inhibitor concentrations in the horse might make the balance between elastases and their inhibitors quite tenuous. Since phenotypes in the equine species have been reported that are missing most of their antichymotrypsin activity [52], and we have found that only inhibitor I is an efficient chymotrypsin inhibitor (results not shown), there is a possibility of a congenital deficiency of the major antielastase (inhibitor I) that cannot be detected by the commonly used immunoelectrophoretic techniques because of immunological identities among all three inhibitors. Such an undetectable deficiency state may account for the common development of lung pathologies in such animals and requires further investigation by more sensitive assays that measure horse serum elastase-inhibitory capacity.

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