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

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INTRODUCTION

Human \( \alpha_1 \)-proteinase inhibitor (\( \alpha_1 \)-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 \( \alpha_1 \)-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 P1 methionine residue of \( \alpha_1 \)-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 \( \alpha_1 \)-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 \( \alpha_1 \)-PI has been previously reported [14,15], together with the suggestion that the deficiency of a specific elastase isoelastator 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.

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 \( \alpha_1 \)-proteinase inhibitor, the reactive-site loops of each of these proteins show extensive variation. Only inhibitor I, with a P1 methionine residue, resembles human \( \alpha_2 \)-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 P1 arginine residue, is an exclusive trypsin inhibitor, and inhibitor III is an oxidation-resistant slow-reacting elastase inhibitor with a P1 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.

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 \( \alpha_1 \)-PI. It consisted of the following steps: (1) (NH₄)₂SO₄ 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 isoelastators 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: \( \alpha_1 \)-PI, \( \alpha_1 \)-proteinase inhibitor.

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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 α1-PI (human leucocyte elastase and horse leucocyte proteinases 2A and 2B). Active-site titration of α1-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-CaCl₂ 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 α1-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 α1-PI-related inhibitors, following the procedure for human α1-PI, resulted in a yield of about 135 mg of protein from 500 ml of plasma. The content of the three different isoforms in the mixture was estimated by titration of both native or oxidized
Table 2. Amino acid sequence of horse plasma inhibitor-target proteinase complexes

<table>
<thead>
<tr>
<th>Residues identified</th>
<th>Trypsin inhibitor (inhibitor II)</th>
<th>Oxidation-sensitive inhibitor (inhibitor I)</th>
<th>Oxidation-resistant inhibitor (inhibitor III)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glu, Ile</td>
<td>Glu, Ile, Thr</td>
<td>Thr</td>
</tr>
<tr>
<td>2</td>
<td>Asp, Val</td>
<td>Asp, Val, Leu</td>
<td>Leu</td>
</tr>
<tr>
<td>3</td>
<td>Pro, Gly</td>
<td>Pro, Gly, Leu</td>
<td>Leu</td>
</tr>
<tr>
<td>4</td>
<td>Glu, Gly</td>
<td>Glu, Gly, His</td>
<td>His</td>
</tr>
<tr>
<td>5</td>
<td>Gly, Tyr</td>
<td>Gly, Tyr, Thr</td>
<td>Thr</td>
</tr>
<tr>
<td>6</td>
<td>Asp, Thr</td>
<td>Asp, Thr, Asn</td>
<td>Asn</td>
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<td>7</td>
<td>Ala</td>
<td>Ala, Glu</td>
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<tr>
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<td>Val, Arg</td>
<td>Val, Arg, Phe</td>
<td>Phe</td>
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<tr>
<td>9</td>
<td>Pro</td>
<td>Pro, Asn</td>
<td>Asn</td>
</tr>
<tr>
<td>10</td>
<td>Glu, His</td>
<td>Glu, His, Arg</td>
<td>Arg</td>
</tr>
</tbody>
</table>

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 FPLC 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 FPLC 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 Mr 52,000 and 53,000 whereas inhibitors II and III gave single bands of Mr 56,000 and 54,000 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 clostridain resulted in rapid loss of inhibitory activity (Figs. 1b and 1a 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 clostridain (Fig. 1a) but was most sensitive for proteolytic cleavage by the V8 proteinase (Fig. 1b). 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 decapetide 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-Tyr-Asp-

Inhibitor II (trypsin inhibitor):

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

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

<table>
<thead>
<tr>
<th>Cycle (inhibitor II)</th>
<th>Arg, Ala</th>
<th>Arg, Met</th>
<th>Arg, Ala</th>
<th>Arg, Ala</th>
<th>Arg, Ala</th>
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<td>His, Ile</td>
<td>His, Pro</td>
<td>His, Pro</td>
<td>His, Pro</td>
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<tr>
<td></td>
<td>Ala, Arg</td>
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<td>Ala, Ala</td>
</tr>
<tr>
<td></td>
<td>Thr, Thr</td>
<td>Thr, Met</td>
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<tr>
<td></td>
<td>Lys, Leu</td>
<td>Lys, Ser</td>
<td>Lys, Leu</td>
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<tr>
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<td>Asp, Leu</td>
<td>Asp, Leu</td>
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<tr>
<td></td>
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<td>Asp, Pro</td>
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<td>Asp, Asn</td>
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<tr>
<td></td>
<td>Asn, Thr</td>
<td>Asn, Pro</td>
<td>Asn, Asn</td>
<td>Asn, Asn</td>
<td>Asn, Asn</td>
</tr>
<tr>
<td></td>
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<td>Glu, Asn</td>
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<tr>
<td></td>
<td>His, Val</td>
<td>His, Leu</td>
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<td>His, Val</td>
<td>His, Val</td>
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<tr>
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<tr>
<td></td>
<td>Gin, Phe</td>
<td>Gin, Phe</td>
<td>Gin, Phe</td>
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<tr>
<td></td>
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<tr>
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<tr>
<td></td>
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<tr>
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<tr>
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<td>Asn, Asp</td>
<td>Asn, Asp</td>
<td>Asn, Asp</td>
<td>Asn, Asp</td>
</tr>
</tbody>
</table>

Inhibitor III (oxidation-resistant elastase inhibitor):

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

Thus MET-SER, ARG-THR and ALA-THR represent the P1–P1 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 clostridain.

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_{on} > 10^3 M^{-1} 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 P1 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 P1 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 α1-PI, oxidative
Horse plasma serpins

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

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

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Oxidation-sensitive elastase inhibitor (inhibitor I)</th>
<th>Oxidation-resistant elastase inhibitor (inhibitor III)</th>
<th>Trypsin inhibitor (inhibitor II)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Native</td>
<td>SucNCl-treated</td>
<td>Native</td>
</tr>
<tr>
<td>Porcine trypsin</td>
<td>1.2 x 10^4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pig pancreatic elastase</td>
<td>*</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>Human leucocyte elastase</td>
<td>6.0 x 10^7</td>
<td>0</td>
<td>3.2 x 10^4</td>
</tr>
<tr>
<td>Proteinase 2A</td>
<td>2.0 x 10^7</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Proteinase 2B</td>
<td>5.0 x 10^7</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Human thrombin</td>
<td>0†</td>
<td>N.D.</td>
<td></td>
</tr>
</tbody>
</table>

* 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.

DISCUSSION

Horse plasma α1-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 iso-inhibitors [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 electrophoreses [15,28–30]. From our preparation of horse α1-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 all closely related to each other (Table 1).

Despite this remarkable identity at the N-terminus, horse α1-PI isoforms differ significantly within the reactive-site loop (Table 5). In this region only inhibitor I demonstrates significant similarity to human α1-PI. Indeed, eight of ten residues between the P_4 and P_3' subsites of the reactive site are identical. This inhibitor, after oxidation, loses all activity against elastases or trypsin (Table 4), whereas human α1-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_3' positions in the horse inhibitor, in comparison with that of the P_4 and P_3 residues in human α1-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_4 residue dictates serpin specificity which is further modulated by adjacent residues, including those in P_3' positions [33], it may well be that, in the case of the horse inhibitor, specificity might be determined by the amino-terminal sequence.
also involve the P\textsubscript{3} and P\textsubscript{4}' 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\textsubscript{3} arginine residue. A comparison of the amino acid sequence at the reactive centre of this inhibitor with human \(\alpha_2\)-PI reveals some similarity, but major differences occur at or near the P\textsubscript{3} 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\textsubscript{3} and P\textsubscript{4}' 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 proteases for C1 inhibitor.

Inhibitor III was determined to have a P\textsubscript{3} 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\textsubscript{3} 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 contraspin-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 \(\alpha_2\)-PI are expressed and present in plasma, and it is likely that genes encoding them were created by ancestral \(\alpha_2\)-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 protease 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 \(\alpha_2\)-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\textsubscript{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 \(\alpha_2\)-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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