HUMAN PLASMA α_1-PROTEINASE INHIBITOR: TEMPORARY INHIBITION AND MULTIPLE MOLECULAR FORMS OF THE COMPLEX WITH PORCINE TRYPSIN*

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Summary: Human plasma α_1-proteinase inhibitor (M.W. 58,000) reacts with porcine trypsin to form a 1:1 complex (M.W. 76,000) which dissociates at pH 8.0 into a modified, inactive inhibitor (M.W. 54,000) and active trypsin. The % recovery of active trypsin decreases with increasing enzyme to inhibitor ratios. Unrecovered trypsin is present in modified, more stable, enzyme-inhibitor complexes.

Human α_1-proteinase inhibitor (α_1-PI) combines with a number of serine proteases to form 1:1 complexes which are stable after reduction and denaturation in Na dodecyl SO_4 (SDS). A modified inhibitor is obtained by dissociation of either the denatured bovine trypsin complex in the presence of hydrazine (1) or the native porcine trypsin complex in the presence of benzamidine (2). In the latter case, active trypsin is recovered. Moroi and Yamasaki (1) showed that the β-trypsin complex (M.W. 74,000) was rapidly degraded in the presence of excess trypsin to proteins of M.W. 63,000, 54,000 and 50,000, and that the inhibition was not temporary.

We now present evidence that a 1:1 complex of α_1-PI and

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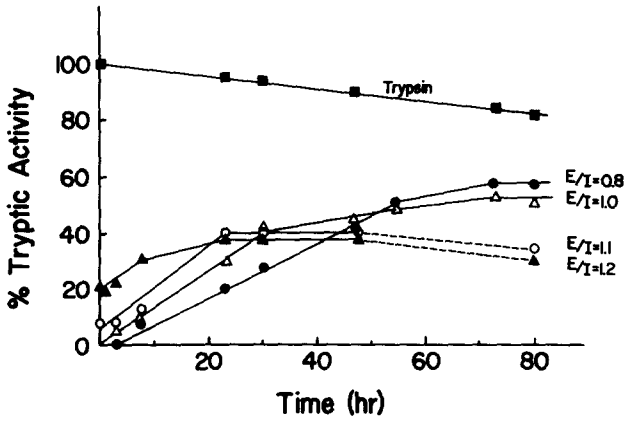

Fig. 1. Effect of E/I ratio on the liberation of tryptic activity. E/I ratios 0.8, 1.0, 1.1 and 1.2 were obtained by combining 211, 264, 290 and 317 µg, respectively, of porcine trypsin with 600 µg α_1-PI in a total volume of 2.0 ml 0.01 M Tris-HCl (pH 8.0), 0.01 M $CaCl_2$. Stoichiometry was determined by titration of trypsin with α_1-PI. The mixtures were incubated at 25-27° C, and aliquots (200 µl) were withdrawn at the indicated times and immediately frozen. After all samples were collected, the samples were thawed and assayed for trypsin activity, which was corrected for losses due to tryptic autolysis in incubations of trypsin alone.

porcine trypsin slowly liberates active trypsin and an inactive, modified inhibitor at pH 8.0. The inhibition appears temporary (3), but recovery of active trypsin is not total due to formation of modified enzyme-inhibitor (E-I) complexes which are more stable than the complex initially formed.

Experimental Procedures

Human α_1-PI was prepared as described (4) except that two chromatography steps were added: 1) DEAE-cellulose at pH 6.5 and 2) Blue Dextran Sepharose (5). The inhibitor (600 units/mg) showed a single band when 25 µg of protein was electrophoresed at pH 7.5 and 9.4 (6), or at pH 7.0 with SDS after reduction and denaturation (7). Porcine trypsin from Novo (ca. 90% active towards p-nitro phenyl-p'-guanido benzoate (8)) was assayed using benzoyl-L-arginine ethyl ester (9). [^{125}I]-trypsin (20µCi/mg)

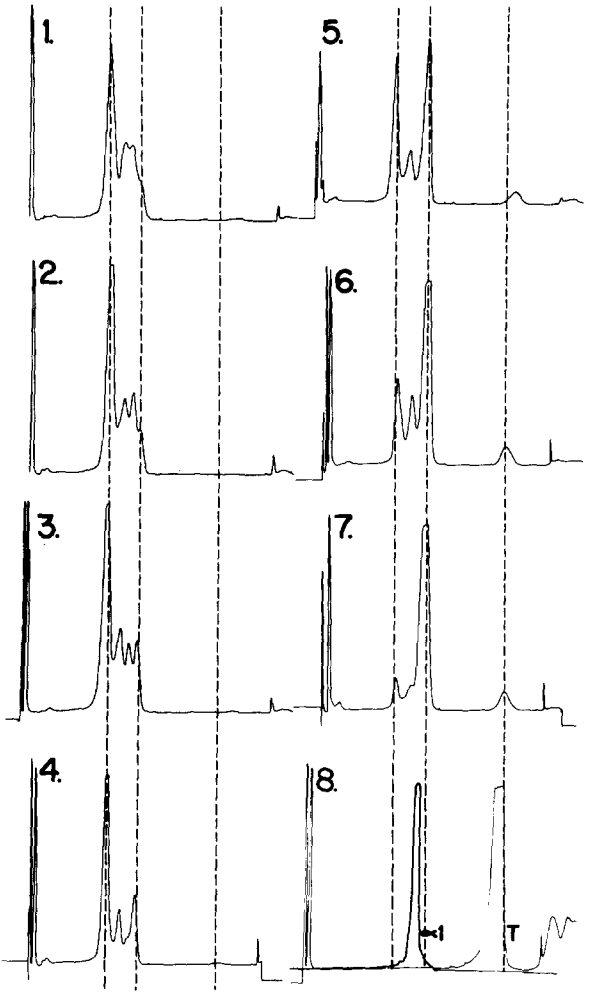

Fig. 2. SDS-polyacrylamide gel analysis of the effects of incubation time on the E/I=1.0 mixture. Aliquots (35 µl) of the E/I=1.0 mixture described in Fig. 1 were treated with 1% 2-mercaptoethanol and 1% SDS, heated 5 min at 100° and electrophoresed on 7.5% acrylamide gels in the presence of SDS. After staining with Coomassie Brilliant Blue and diffusion destaining, the gels were scanned using a Gilford spectrophotometer equipped with a linear transport. The dashed vertical lines correspond from left to right to the positions of proteins of M.W. 76,000, 54,000 and 23,000. Panel 8 is a composite of two scans, α_1-PI alone and trypsin alone. Panel 1 - zero time, 2 - 1 hr, 3 - 3 hr, 4 - 7 hr, 5 - 23 hr, 6 - 30 hr, 7 - 47 hr.

was prepared according to McConahey and Dixon (10). Bovine pancreatic trypsin inhibitor (Kunitz) was prepared (11) and activity determined as described (4).

Results and Discussion

As shown in Fig. 1, at E/I=0.8, ca. 60% of the trypsin is ultimately released from the E-I complex, suggesting that α_1-PI is a temporary inhibitor of porcine trypsin. At E/I > 0.8, however, the % tryptic activity released decreases. At E/I=1.2, only ca. 20% is released. The addition of 1.5 equivalents of pancreatic trypsin inhibitor (PTI) to the E/I=1.0 mixture at zero time did not affect the rate or extent of liberation of active trypsin (measured as unreacted PTI). Thus, the nature of the inhibition (temporary or permanent) of porcine trypsin by α_1-PI appears to depend on the initial E/I ratio, with smaller ratios favoring temporary inhibition. This conclusion reconciles our findings with those of Moroi and Yamasaki (1), who tested for temporary inhibition at E/I=1.3 and ignored the small, transient increase in tryptic activity.

To account for incomplete release of active trypsin, experiments were performed in which aliquots of the enzyme-inhibitor incubation mixture were reduced, denatured in SDS, and electrophoresed in 7.5% acrylamide gels (7). Shown in Fig. 2 are the scans of the Coomassie-stained gels of an experiment in which E/I=1.0. Four peaks ranging from 76,000 to 54,000 daltons are seen. The E-I complex of M.W. 76,000 (peak 1) slowly dissociates and gives rise to peak 4 (M.W. 54,000) and trypsin (M.W. 23,000). The material in peak 4 is inactive against trypsin and appears resistant to tryptic digestion since it accumulates simultaneously with active trypsin. Peak 3 is uncomplexed α_1-PI (M.W. 58,000) which decreases between 0 and 7 hr (panels 1-4) due to complexation with liberated trypsin. Peak 2 (M.W. 64,000) remains fairly constant during the first 7 hr of incubation (panels 1-4). Between 7 and 23 hr, the material in this peak decreases slightly

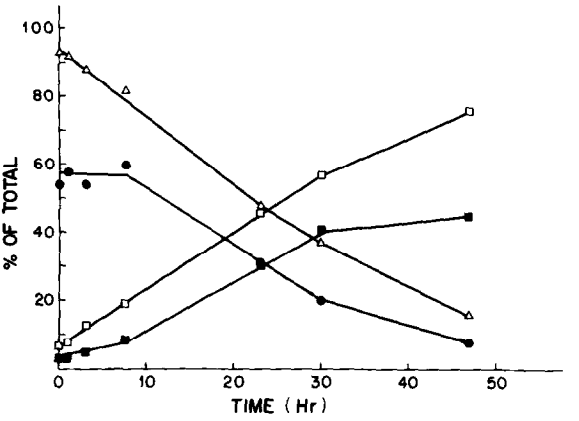

Fig. 3. Effects of incubation time on peak intensities in Fig. 2 and the tryptic activity. The areas under peaks 1, 2+2'+3, and 4 shown in Fig. 2 were determined by cutting out the peaks and weighing them. Plotted is % of total area: Δ-Δ, the sums of peaks 1, 2, 2' and 3; □, peak 4; ● - ●, peak 1. Trypsin activity (■ - ■) is plotted as % of total tryptic activity corrected for autolysis. It was assumed that the staining of the bands 1-4 is determined primarily by the α_1-PI component since an equimolar complex of α_1-PI and porcine trypsin contains a 2.5 fold weight excess of α_1-PI over trypsin.

in M.W. We will refer to this lower M.W. material as peak 2' (M.W. 61,000). Between 23 and 47 hr, peak 2' decreases (panels 5-7).

The areas under the peaks 1, 2+2'+3, and 4 were determined for the scans shown in Fig. 2 and plotted as % of total area (Fig. 3). The % of total tryptic activity released is also shown. The decrease in peak 1 corresponds to the increase in active trypsin. This suggests that the M.W. 76,000 complex is responsible for the observed temporary inhibition and dissociates to give active trypsin and inactive α_1-PI. The lag in the disappearance of peak 1 and in the appearance of trypsin is due to ca. 10% uncomplexed α_1-PI (peak 3). Peak 4 increases as a result of the decrease in peaks 1+2+2'+3. Most of peak 4 is inactive, modified α_1-PI (α_1-PI*). The M.W. of α_1-PI* (54,000) is lower than that of α_1-PI by ca. 4,000. Presumably, trypsin

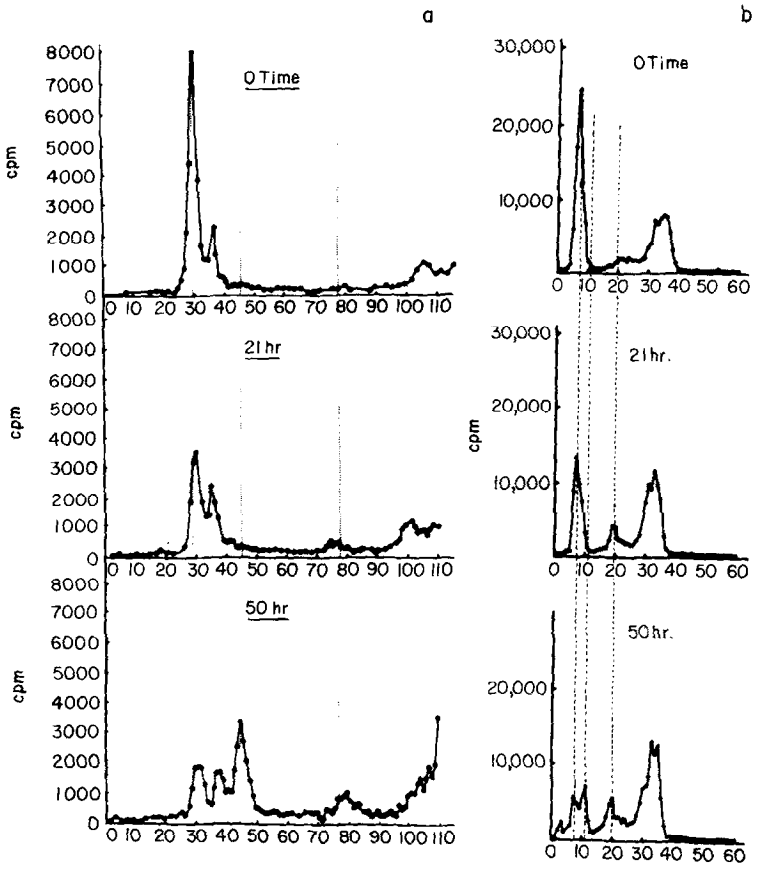

Fig. 4. SDS-polyacrylamide gel analysis of the effects of incubation time on the complex of α_1-PI with [^{125}I]-trypsin. E/I= 1.0 was obtained by combining 1800 µg α_1-PI with 846 µg [^{125}I]-trypsin (20µCi/mg) in 1.5 ml 0.05 M Tris-HCl (pH 8.0), .01 M $CaCl_2$. During the 25° C incubation, aliquots were withdrawn at the indicated times and frozen immediately. Samples were prepared for electrophoresis in a) 7.5% acrylamide (25 µl samples) and b) 15% acrylamide (50 µl samples) gels as described in Fig. 2. The gels were sliced into 0.5 mm segments (4 drops/fraction) using a Gilford gel slicer. Radioactivity in the fractions was counted using a Packard auto-gamma spectrometer. The dashed vertical lines correspond from left to right to the positions of M.W. 76,000, 54,000 and 23,000.

activity not recovered in tests for temporary inhibition is present in peaks 1 and 2' (Fig. 2, panel 6) and this material also decomposes to give peak 4 (Fig. 2, panel 7), which is then a mixture of products.

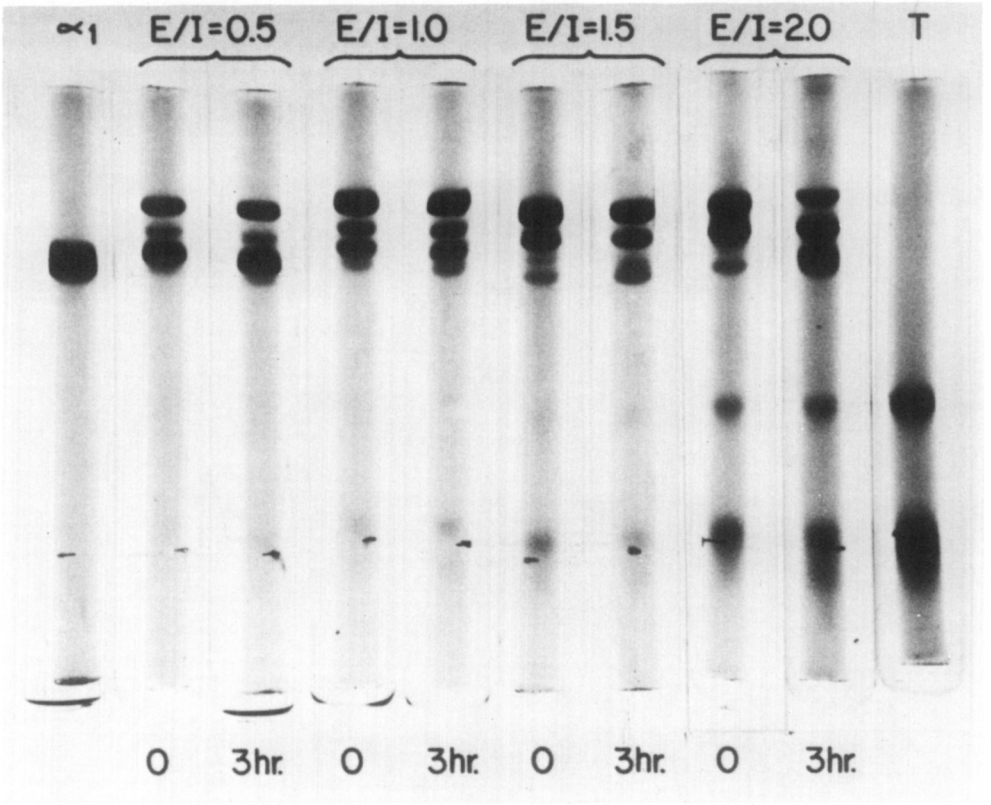

Fig. 5. Effect of E/I on SDS-polyacrylamide gel patterns. E/I ratios 0.5, 1.0, 1.5 and 2.0 were obtained by combining 74, 148, 221 and 294 µg, respectively, of porcine trypsin with 300 µg α_1-PI in 0.6 ml 0.01 M Tris-HCl (pH 8.0), 0.01 M $CaCl_2$. Aliquots were withdrawn at 0 and 3 hr (25° C) and frozen immediately. Samples (25 µl) were prepared for electrophoresis on 7.5% acrylamide gels as described in Fig. 2. In the gel shown for E/I=1.0 (3 hr), the bands (from top to bottom) represent protein of the following M.W.: 1 - 76,000, 2 - 64,000, 3 - 58,000 and 4 - 54,000.

To test this hypothesis, the dissociation of the complex of α_1-PI with [^{125}I]-trypsin (E/I=1.0) was monitored by gel electrophoresis in SDS. At zero time, [^{125}I]-trypsin is present in peaks 1 and 2 (Fig. 4), showing the existence of at least two types of E-I complex. At 21 hr, the decrease in peak 1 is accompanied by an increase in free trypsin and trypsin autolysis products (Fig. 4). Little change in intensity of peak 2' is

evident at 21 hr. The distribution of [^{125}I]-trypsin in peaks 1 and 2 or 2' at zero and 21 hr is similar to the distribution of protein (Fig. 2, panels 1 and 5). At 21 hr, no [^{125}I]-trypsin is present in peak 4, α_1-PI* (compare Fig. 2, panel 5). After 21 hr, peak 4 increases in radioactivity at the expense of peaks 1 and 2' (Fig. 4, 50 hr). Thus at 50 hr, at least three types of E-I complexes exist. At least two of these complexes (peaks 2' and 4) are modified by proteolytic digestion of the trypsin and/or α_1-PI since their molecular weights (peak 2', 61,000; peak 4, 54,000) are less than that of the complex of M.W. 76,000 (peak 1). We can not rule out the possibility that the material in peak 1 at times > 21 hr is also modified. Thus, unrecovered trypsin is contained in modified E-I complexes which are apparently more stable than the 76,000 dalton E-I complex.

We would expect that under conditions in which the recovery of active trypsin is decreased (E/I > 1.0), an increase in the proportion of the modified E-I complexes should be observed. Fig. 5 shows an experiment in which E/I was varied between 0.5 to 2.0 and samples of the mixture were electrophoresed (7) after 0 and 3 hr incubations. At zero time, the M.W. 76,000 E-I complex (band 1) increases at the expense of α_1-PI (band 3, M.W. 58,000) as E/I is increased. Similarly, the modified E-I complex of M.W. 64,000 (band 2) increases. For all E-I ratios tested, the proportion of α_1-PI* (band 4, M.W. 54,000) is low. After 3 hr, the material in band 4 increases dramatically with increasing E/I. At E/I=2.0, band 4 increases in intensity while band 1 decreases. Under these conditions, band 4 is mostly modified E-I complex and not α_1-PI*, since there is little or no increase in free trypsin. Thus, the results shown in Fig. 5 support the contention that modified E-I complexes (bands 2 and 4) increase in amounts with increasing E-I ratios.

Since these modified E-I complexes were observed on gels after denaturation and reduction of the proteins, their molecular sizes prior to this treatment could actually be much larger. Indeed, without reduction, the M.W. 64,000 complex co-migrates with the M.W. 76,000 complex. Moroi and Yamasaki (1) showed that the complex of α_1-PI with bovine α-trypsin migrated as protein of M.W. 63,000 after reduction and M.W. 74,000 prior to reduction. Thus, the modification which produces band 2 is probably the conversion of β- to α-trypsin.

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