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THE TRYPSIN-INHIBITORY EFFICIENCY OF HUMAN α_2-MACROGLOBULIN IN THE PRESENCE OF α_1-PROTEINASE INHIBITOR: EVIDENCE FOR THE FORMATION OF AN α_2-MACROGLOBULIN–α_1-PROTEINASE INHIBITOR COMPLEX

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The inhibition of bovine pancreatic trypsin was studied at pH 7, 25°C, using mixtures of purified human α_2-macroglobulin (α_2M) and α_1-proteinase inhibitor (α_1PI). The partitioning of the enzyme between the two inhibitors was determined by comparing control esterase activity, assayed with N-benzoyl-L-arginine ethyl ester as substrate, with that remaining after incubation with inhibitory mixtures. (At $[I]_0 > [E]_0$, remaining esteratic activity reflects the concentration of α_2M-associated enzyme (α_2M–E*) and the concentration of α_1PI-associated, inactive enzyme (α_1PI–E*) is given by the difference, $[E]_0 - [\alpha_2\text{M–E*}]$.) The pattern of product distribution was found to be incompatible with an inhibitory model involving parallel, second-order reactions of E with α_2M and α_1PI. The data pointed to complex formation between the two inhibitors, limiting the level of α_2M readily available for reaction with E. Analysis based on the binding equilibrium, α_2M (dimeric unit) + α_1PI $\rightleftarrows \alpha_2$M – α_1PI, yielded $K_d = 2.1 \pm 0.3\,\mu$M. Complex formation between α_2M and α_1PI was verified by gel permeation experiments. α_2M was found to restrict the volume of distribution of α_1PI in Sephadex G200 beds. K_d, deduced from gel permeation behaviour, was $0.8 \pm 0.32\,\mu$M. Preliminary kinetic experiments with dialyzed plasma suggested that the α_2M–α_1PI interaction is effective also *in vivo*. Given K_d and the mean plasma levels of the two inhibitors ($[\alpha_2\text{M}] = 2\,\mu$M; $[\alpha_1\text{PI}] = 36\,\mu$M), it was estimated that $> 90\%$ of α_2M in human circulation must be complexed to α_1PI and lack immediate antiproteinase activity.

Keywords: α_2-Macroglobulin; α_1-Proteinase inhibitor; Protein–protein interaction

Abbreviations: α_2M, α_2-macroglobulin; α_1PI, α_1-proteinase inhibitor; α_2M–E*, entrapment complex between trypsin and α_2M; α_1PI–E*, stable, covalent complex of trypsin and α_1PI; $[E]_0$ and $[I]_0$, total concentrations of enzyme and inhibitor at zero time; $[E]_t$ and $[I]_t$, total

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concentrations of enzyme and inhibitor at time t; KP, 20 mM potassium phosphate buffer, pH7

INTRODUCTION

α_2-Macroglobulin and α_1-proteinase inhibitor are two plasma proteins which participate in the binding and clearance of proteases in mammalian circulation.[1–7] Both inhibitors have been shown to act essentially as suicide substrates. With α_2M, proteolytic cleavage of bait regions in the 725 kDa tetrameric molecule leads to a conformational change, which results in physical entrapment of the protease and steric inhibition of enzymatic activity towards macromolecular subtrate.[3,7] With α_1PI, the inhibitory process also starts as a proteolytic cycle, targeting the reactive site loop of the 51 kDa inhibitor. However, the inhibition in this case is based on a covalent interaction between E and I; the catalytic cycle is thwarted by the formation of a stable I–E* species which has a physiologically negligible turnover rate.[1,8,9] Both the α_2M–E* inclusion complex and the α_1PI–E* covalent complex are recognized and cleared by endocytotic receptors.[5,10,11]

The kinetics of inhibition of a number of proteases by purified α_2M and α_1PI have been determined.[12–18] Considering the plasma concentrations of the two inhibitors and the second-order rate constants (k_{ass}) for protease inhibition, it has been suggested that α_2M is a non-specific protease inhibitor.[1] α_1PI, on the other hand, emerges as the principal scavenger of neutrophil elastase *in vivo*. Applying the kinetic data relating to α_2M and α_1PI in isolation to mixtures of the two inhibitors, it may be estimated that *ca.* 80% of trypsin (as a "general purpose protease") will be trapped as α_2M–E*; *ca.* 70% of neutrophil elastase will be transformed into α_1PI–E*.

The present study concerns the actual fate of trypsin in mixtures of purified α_2M and α_1PI. The partitioning of the enzyme between the two inhibitors and, thereby, the effective ratio of the second-order rate constants ($k_{\alpha_2M}/k_{\alpha_1PI}$) for the association of the protease with α_2M and α_1PI have been determined. We find that the observed value of $k_{\alpha_2M}/k_{\alpha_1PI}$ is different from the expected value, and that it varies as a function of $[I]_0$, $[E]_0$ and the inhibitor ratio. The results point to a binding equilibrium between α_2M and α_1PI, which limits the inhibitory potential of α_2M (if not both inhibitors). The effect of α_2M on the gel permeation behaviour of α_1PI also suggests a complex formation between the two proteins.

MATERIALS AND METHODS

Citrated blood samples were obtained from the blood bank of Hacettepe University Hospitals before the expiration date for clinical use, dialyzed against 20 mM potassium phosphate buffer, pH 7, and stored at 4°C with 0.02% sodium azide as preservative. Chromatographic matrices and biochemicals were obtained from Sigma (St. Louis, Mo, USA). All other chemicals were from Sigma or Merck (Darmstadt, Germany) and were of the highest purity available. Stock solutions of N-tosyl-L-phenylalanine chloromethyl ketone-treated bovine pancreatic trypsin were prepared in 2 mM HCl. The concentration of active enzyme was determined by titration with *p*-nitrophenyl, *p'*-guanidinobenzoate.[19]

Purification of Inhibitors

α_2M was purified essentially as described previously.[20] α_1PI was purified by consecutive chromatographic steps using: (a) DEAE-Trisacryl M and a linear gradient of 0–0.25 M KCl in KP, (b) Polybuffer Exchanger 94 and a linear gradient of 0–0.2 M KCl in imidazole-HCl buffer, pH 6.2, and (c) Cibacron Blue-Agarose, equilibrated and eluted with KP.

The purity of the preparations was verified by SDS-PAGE under reducing condition.[21] α_1PI (specific activity, 11–15 nmol trypsin inhibited/mg protein) revealed an accessory band corresponding to partially proteolyzed inhibitor. Both preparations were stored at 4°C in KP containing 0.02% sodium azide. The stock solution of α_1PI further contained 0.2 mM EDTA to prevent progressive inactivation due to proteolysis.

Protein Determination

Protein concentrations in purified stock solutions were calculated from A_{280}. Extinction coefficients and M_r values used were: α_2M, $0.901 \cdot g^{-1} \cdot cm^{-1}$ and 725 000 Da[16]; α_1PI, $0.551 \cdot g^{-1} \cdot cm^{-1}$ and 51 000 Da.[22]

Assay of Inhibitor Activity

All assays were performed at 25°C. α_1PI was assayed by preincubating 1 μM trypsin with an appropriate amount of the inhibitor for 2 min in KP. Residual tryptic activity was determined by adding a 200-μl aliquot of the preincubation mixture to 1 ml 10 mM Tris-HCl, pH 8, containing 0.5 mM N-benzoyl-L-arginine ethyl ester as substrate. The hydrolytic reaction was monitored at 253 nm. Enzyme 1 μM in the final assay mixture caused an

absorbance change of 0.74 ± 0.085 OD units min^{-1}; the concentration of α_1PI was calculated from the difference between $\Delta A\ min^{-1}$ observed in the absence and presence of inhibitor.

To assay for α_2M, the samples (*ca.* 0.6 μM trypsin entrapment capacity) were first incubated with 1 μM trypsin for 2 min. Excess exogenous α_1PI was then added, and the preincubation was continued for a further 2 min, to inhibit enzyme not entrapped by α_2M. The α_2M-associated esteratic activity of trypsin was measured as described above. Inhibitor concentration (in terms of μM entrapped trypsin) was calculated from Equation (1). (The factor, 0.74, relates $\Delta A\ min^{-1}$ to μM free enzyme; 1.12 corrects for the difference between the esteratic activities of free and α_2M-bound trypsin, as determined in the present study),

$$[\alpha_2M], \mu M = [\alpha_2M{-}E^*] = \Delta A\ min^{-1} \div (0.74 \times 1.12). \quad (1)$$

In assays for α_2M in mixtures which contained α_1PI as well as α_2M, esteratic activity was determined following preincubation of a fixed volume of sample with increasing amounts of enzyme. Observed esteratic activity increased and reached a plateau at $[\alpha_2M] < [E] < ([\alpha_2M] + [\alpha_1PI])$. $[\alpha_2M]$ was estimated by using the plateau activity and Equation (1).

Titration of Trypsin with Mixtures of Purified Inhibitors

α_2M and α_1PI were mixed in a ratio, $\alpha_2M : \alpha_1PI = 1:5$ upto $1:9$ (in terms of trypsin entrapment or inhibitory activity). A fixed concentration of trypsin (normally *ca.* 1 μM) was preincubated with increasing aliquots of the mixture for 2 min and residual esteratic activity was measured as above.

Study of Gel Permeation Behaviour

A 1 ml sample of inhibitor(s) in KP was added to 3 ml Sephadex G200 equilibrated with KP and allowed to settle under gravity in a graduated test tube. The tube was incubated for 1.5 h, with periodic mixing by inversion. The gel was allowed to pack again and then the supernatant was withdrawn and assayed for inhibitor content.

The parameters relating to the gel matrix were determined by using α_1PI and α_2M in isolation. The total fluid volume (v_{total}) of the system, estimated by comparing the concentrations of α_1PI in the ingoing sample and in the Sephadex G200 supernatant, was 3.1 ± 0.08 ml. The outer volume (v_{outer}), as derived from the concentrations of α_2M in sample and supernatant, was

1.9 ± 0.05 ml. Hence the effective inner (i.e. pore) volume for α_1PI ($v_{inner} = v_{total} - v_{outer}$) was 1.2 ± 0.09 ml.

The distribution of α_1PI into v_{outer} and v_{inner} in mixtures of α_2M and α_1PI was determined as follows. An aliquot of the Sephadex G200 supernatant was titrated with trypsin. The equivalence point (see Figure 5), which yielded the combined trypsin entrapment/inhibitory capacity due to α_2M and α_1PI, was corrected for the contribution of α_2M (as determined in estimations of v_{outer}); the resulting inhibitor capacity was taken to reflect [α_1PI] in the outer volume ([α_1PI]$_{outer}$). The concentration of α_1PI in the inner volume ([α_1PI]$_{inner}$) was in turn calculated from the relationship, v_{total} [α_1PI]$_{total}$ = v_{outer}[α_1PI]$_{outer}$ + v_{inner}[α_1PI]$_{inner}$. The term, [α_1PI]$_{total}$, refers to the concentration of α_1PI in the Sephadex G200 supernatant of the single-component sample used to determine v_{total}.

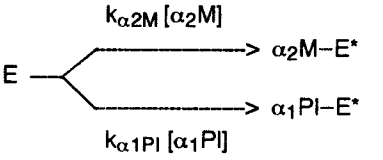
RESULTS AND DISCUSSION

The titration of trypsin with mixtures of α_2M and α_1PI yielded a triphasic curve (Figure 1). Phase (i) covered the region where [α_2M] $\ll$ [E] $>$ [α_1PI], so that α_1PI was the principal inhibitor of E; phase (ii) reflected an increasingly significant contribution by α_2M to the inhibitory process and phase (iii) revealed the beginnings of an apparent reversal of the relative efficiencies of α_2M and α_1PI in the competition for available enzyme. The pattern was similar to that observed in titrations of trypsin with plasma, except for contributions in the latter from additional inhibitors such as inter-alpha-trypsin inhibitor and α_2-antiplasmin.[1]

The data were tested for conformity to feasible kinetic models:

I. Parallel, Second-order Inhibition of *E* by α_2M and α_1PI

The partitioning of proteases between α_2M and α_1PI has previously been analyzed in terms of a kinetic model involving parallel, second-order reactions (Scheme I).[15] This model requires that the rate constants for the association of E with α_2M and α_1PI and the distribution of E into α_2M–E* and



SCHEME I

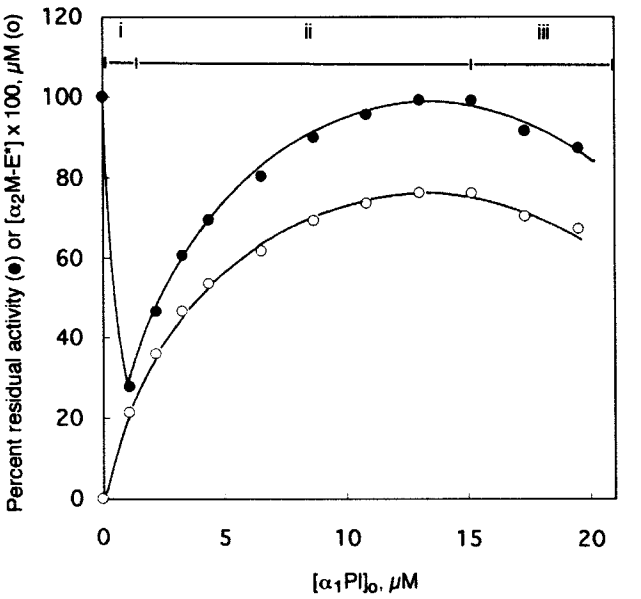


FIGURE 1 Titration of trypsin with a mixture of α_2M and α_1PI. $[\alpha_1PI]_0:[\alpha_2M]_0 = 5.8$; $[E]_0 = 0.86\,\mu M$. The inhibitor ratio is based on the actual trypsin inhibitory or entrapment capacities of the preparations. $[E]_0$ stands for the concentration of active enzyme; (●), Percent residual activity; (○) $[\alpha_2M-E^*]$. Each point is the average of three determinations, which agreed within ±5%

α_1PI–E* be related as in Eq. (2),

$$\frac{k_{\alpha_2M}}{k_{\alpha_1PI}} = \frac{\ln([\alpha_2M]_0/([\alpha_2M]_0 - [\alpha_2M-E^*]_t))}{\ln([\alpha_1PI]_0/([\alpha_1PI]_0 - [\alpha_1PI-E^*]_t))}. \quad (2)$$

As seen in Figure 2, the data failed to conform to this equation. Features pointing against Scheme I were (a) the non-zero ordinate intercept, (b) the low value of $k_{\alpha_2M}/k_{\alpha_1PI}$ (*ca.* 10) derived from the slope, as well as (c) the deviation from linearity at high inhibitor concentration.

A point-by-point evaluation of $(k_{\alpha_2M}/k_{\alpha_1PI})^{apparent}$ as a function of $[\alpha_1PI]_0$ revealed a bell-shaped dependence (Figure 3), passing through a maximum of 55 – a value in reasonable agreement with those obtained by taking the ratio of the second-order rate constants for the association of trypsin with α_2M and α_1PI in isolation (30[12,16]; 150[13,16]; 80 [this study, data not shown]). The inconstancy of the rate constant ratios calculated according to Equation (2) indicated that the reactions of α_2M and α_1PI with trypsin were not simple, parallel processes.

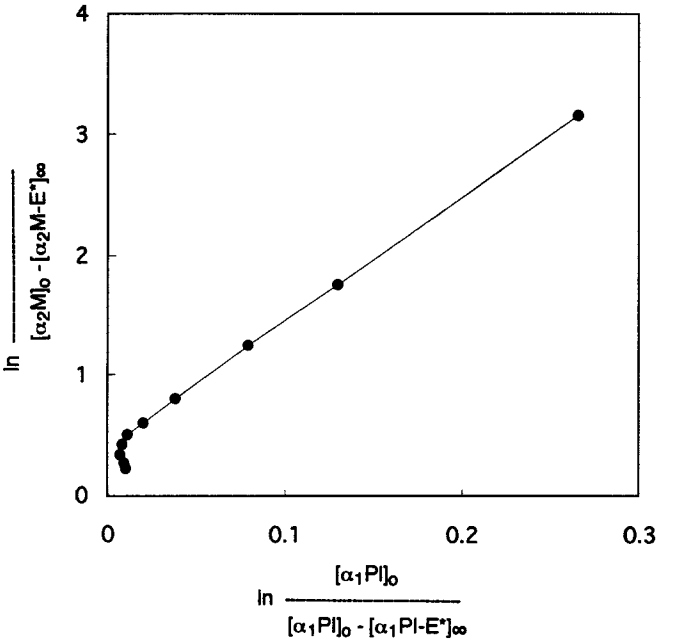


FIGURE 2 Logarithmic plot of the partitioning of trypsin between α_2M and α_1PI according to Equation (2) data as in Figure 1.

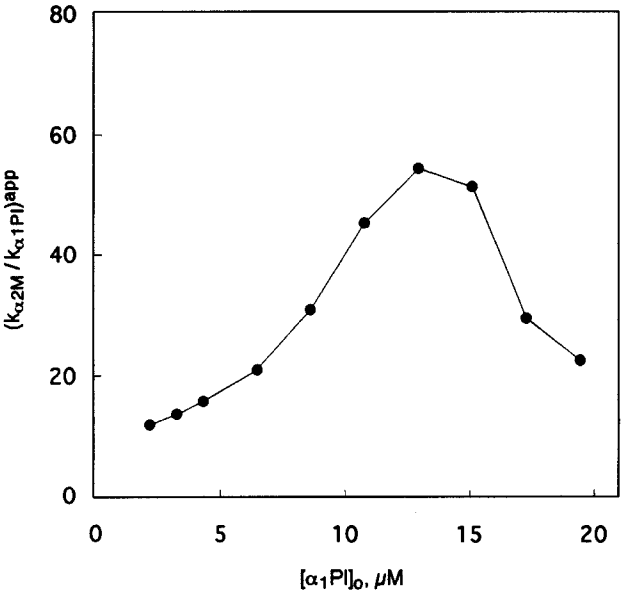


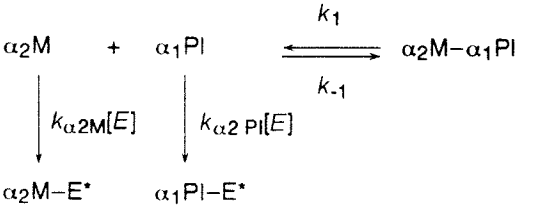
FIGURE 3 Dependence of $(k_{\alpha_2M}/k_{\alpha_1PI})^{app}$ on total inhibitor concentration. Data as in Figure 1; calculations based on Equation (2).

II. Sequential Clearance of E by α_2M and α_1PI

Considering the relative magnitudes of $k_{\alpha_2 M}$ and $k_{\alpha_1 PI}$ ($k_{\alpha_2 M}/k_{\alpha_1 PI} > 30$), it appeared feasible that the results of the titration of a fixed amount of enzyme with inhibitory mixtures might reflect not a true kinetic partitioning, but the end-point of a sequential process: α_2M could stoichiometrically react with E in the early phase of the 2-min preincubation period (see Methods) and α_1PI could subsequently scavenge E in excess of α_2M. While this simplistic model was in keeping with the observed increase in $(k_{\alpha_2 M}/k_{\alpha_1 PI})^{app}$ as $[\alpha_2 M]_0$ approached $[E]_0$ (Figure 3), it failed to account for the reversal of the trend at higher inhibitor concentrations. Furthermore, the maximum concentration of α_2M–E* reached in the titrations was always lower than $[E]_0$ and varied inversely with $[\alpha_1 PI]/[\alpha_2 M]$. Thus α_1PI could not be a strictly passive component of the inhibitory system.

III. Semi-sequential Clearance of E by α_2M and α_1PI; Available α_2M Limited by α_1PI

The discrepancies between the data and the models considered above suggested that α_2M and α_1PI were interacting in mixture and that this interaction decreased the effective concentration of α_2M. A minimal model incorporating these features is given in Scheme II. The scheme proposes that α_2M and α_1PI associate to form a complex, α_2M–α_1PI,



SCHEME II

which has no inhibitory activity. Free and complexed inhibitors come to equilibrium before the addition of enzyme to the preincubation mixture (during the period allowed for temperature equilibration), such that only a fraction of total α_2M is readily available for reaction with E. Hence the partitioning of E between α_2M and α_1PI will be governed by two types of reaction: (a) The reactions of E with free α_2M and free α_1PI and (b) the reactions of E with α_2M and α_1PI generated from the α_2M–α_1PI complex.

In the present study where $[\alpha_2M]_0 \ll [\alpha_1PI]_0$, the concentration of free α_1PI at any time was assumed to approximate $[\alpha_1PI]_t$, the total concentration remaining at that time. The total pool of unreacted α_2M, $[\alpha_2M]_t$, on the other hand, was taken to consist of free (α_2M_{free}) and complexed (α_2M_{bound}) components.

Two ranges of total inhibitor concentration ($[I]_0$) were considered:

(a) Low $[I]_0 : [\alpha_1PI]_0 > [E]_0 > [\alpha_2M]_0$; $[\alpha_2M]_{free} \approx [\alpha_2M]_t$. In this range where $k_{\alpha_2M}[\alpha_2M]_{free,o} \approx k_{\alpha_2M}[\alpha_2M]_0 \gg k_{\alpha_1PI}[\alpha_1PI]_0$, the system should approximate Model II. The initial phase of the preincubation period should involve the near-quantitative conversion of α_2M to $\alpha_2M{-}E^*$ (Equation (3); $[E] = \text{fn}[E]_0, t$); the second phase should involve the clearance of E in excess of α_2M by α_1PI (Equation (4); $[E]_\delta = \text{fn}[E_0 - \alpha_2M_0], t$),

$$\alpha_2M \xrightarrow[[E]]{k_{\alpha_2M}} \alpha_2M{-}E^* \tag{3}$$

$$\alpha_1PI \xrightarrow[[E_\delta]]{k_{\alpha_1PI}} \alpha_1PI{-}E^*. \tag{4}$$

Analysis of such a sequential process according to Equation (2) (which relates to parallel second-order reactions) would lead to an underestimation of $k_{\alpha_2M}/k_{\alpha_1PI}$. i.e. $(k_{\alpha_2M}/k_{\alpha_1PI})^{app} < (k_{\alpha_2M}/k_{\alpha_1PI})^{true}$. The difference between the true and observed values would be expected to depend on the relative magnitudes of $[E]_0$ and $[\alpha_2M]_0$ and should decrease as $[\alpha_2M]_0$ approaches $[E]_0$. The ascending limb of the bell-shaped curve in Figure 3 bore out this prediction.

(b) High $[I]_T : [\alpha_1PI]_0 > [\alpha_2M]_0 > [E]_0$; $[\alpha_2M]_{free,o} < [\alpha_2M]_0$. If the binding equilibrium in Scheme II is valid, increasing total inhibitor concentration should limit the concentration of α_2M free to compete with α_1PI. Under conditions of $[\alpha_1PI]_0 \gg [\alpha_2M]_0$,

$$[\alpha_2M]_{free,o} \approx \frac{K_d}{K_d + [\alpha_1PI]_0}[\alpha_2M]_0 \tag{5}$$

($K_d = k_{-1}/k_1$). Moreover, at $[\alpha_1PI]_0 \gg K_d$, the initial concentration of α_2M_{free} becomes constant (Equation (6)), while $[\alpha_1PI]_{free,o} \approx [\alpha_1PI]_0$ continues to increase linearly with $[I]_0$,

$$[\alpha_2M]_{free,o} \approx K_d \frac{[\alpha_2M]_0}{[\alpha_1PI]_0} \approx \text{constant}. \tag{6}$$

Hence, at high enough $[I]$ the relationship $k_{\alpha_2M}[\alpha_2M]_{free,o} \gg k_{\alpha_1PI}[\alpha_1PI]_0$, should no longer be valid and the partitioning of E between the inhibitors should be governed by three parallel second-order reactions (Equations (7)–(9)).

$$\alpha_2M_{free} \xrightarrow[[E]]{k_{\alpha_2M}} \alpha_2M{-}E^*_{(i)} \tag{7}$$

$$\alpha_2M_{bound} \xrightarrow[[E]]{k'_{\alpha_2M}} \alpha_2M{-}E^*_{(ii)} \tag{8}$$

$$\alpha_1PI \xrightarrow[[E]]{k_{\alpha_1PI}} \alpha_1PI{-}E^* \tag{9}$$

(The subscripts, (i) and (ii), have been introduced to differentiate between the two sources of $\alpha_2M{-}E^*$; k'_{α_2M} is a steady-state constant (see below).) In this case $(k_{\alpha_2M}/k_{\alpha_1PI})^{app}$, calculated according to Equation (2) will reflect not true k_{α_2M}, but an effective rate "constant", sensitive to the $[I]_0$-dependent variation in the relative contributions of Equations (7) and (8) to the overall inhibition of E by α_2M. If $k'_{\alpha_2M} < k_{\alpha_1PI}$, an increase in $[I]_0$ (hence in $[\alpha_2M]_{bound}$ relative to $[\alpha_2M]_{free}$) should result in a decrease in $(k_{\alpha_2M}/k_{\alpha_1PI})^{app}$, as the contribution of Equation (7) to the partitioning of E diminishes. The lower limit for $(k_{\alpha_2M}/k_{\alpha_1PI})^{app}$ at constant $[\alpha_2M]_0 : [\alpha_1PI]_0$ and $[E]_0$ is $k'_{\alpha_2M}/k_{\alpha_1PI}$.

The descending limb of the curve in Figure 3 supported the predictions concerning the inhibitory process at high $[I]_0$. Thus the data were in qualitative agreement with Model III over the inhibitor concentration range covered. (An alternative model involving dimerization or aggregation of α_2M, rather than the formation of an $\alpha_2M{-}\alpha_1PI$ complex, could also be proposed. This possibility was dismissed for lack of evidence: The second-order rate constant for the association of trypsin with α_2M in isolation is independent of inhibitor concentration in the 1–10 μM range ($1–2 \times 10^7\ M^{-1}\ s^{-1}$).[16,23] We have determined a value of $2.1 \pm 0.6 \times 10^7\ M^{-1}/s^{-1}$ at submicromolar $[\alpha_2M]$. Self-association, if present, does not appear to affect inhibitory efficiency.)

Additional Considerations Relating to Model III

In the high-$[I]_0$ range where $(k_{\alpha_2M}/k_{\alpha_1PI})^{app} \approx k'_{\alpha_2M}/k_{\alpha_1PI}$, an additional feature of Model III should become apparent: At constant $[\alpha_2M]_0$ and $[\alpha_1PI]_0$, $(k_{\alpha_2M}/k_{\alpha_1PI})^{app}$, as calculated according to Equation (2) should vary as an

inverse function of $[E]_0$. This property derives from the fact that k'_{α_2M} is a steady-state constant (Equation (10)):

$$d[\alpha_2M{-}E^*_{(ii)}]/dt = k_{\alpha_2M}[E]_t[\alpha_2M_{(ii)}]_{ss}$$

$$= \frac{k_{-1}k_{\alpha_2M}[E]_t[\alpha_2M]_t}{k_1[\alpha_1PI]_t + k_{\alpha_2M}[E]_t}$$

$$k'_{\alpha_2M} = \frac{k_{-1}k_{\alpha_2M}}{k_1[\alpha_1PI]_t + k_{\alpha_2M}[E]_t} \qquad (10)$$

$([\alpha_2M_{(ii)}]_{ss}$ = steady-state level of free α_2M derived from $\alpha_2M{-}\alpha_1PI$

$$= \frac{k_{-1}[\alpha_2M]_{bound}}{k_1[\alpha_1PI]_{free} + k_{\alpha_2M}[E]_t}$$

$$\approx \frac{k_{-1}[\alpha_2M]_t}{k_1[\alpha_1PI]_t + k_{\alpha_2M}[E]_t}\Bigg).$$

The observed relationship between $(k_{\alpha_2M}/k_{\alpha_1PI})^{app}$ and initial enzyme concentration at constant initial inhibitor composition is given in Figure 4. The data provide further support for the formation of an $\alpha_2M{-}\alpha_1PI$ complex.

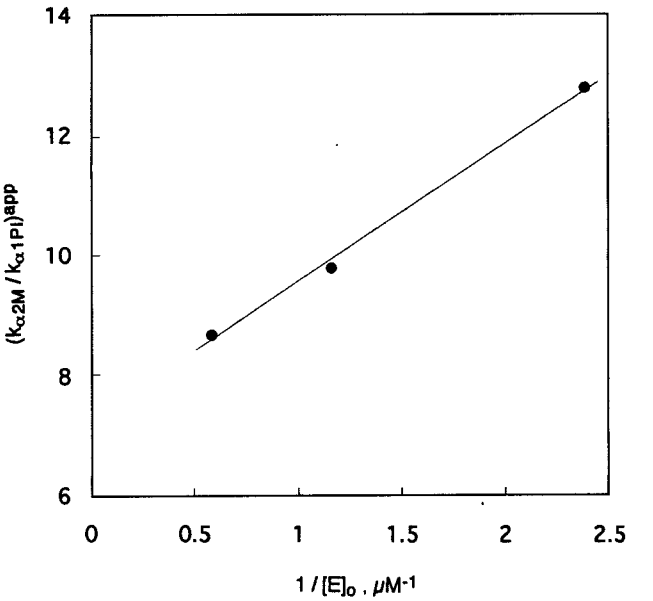


FIGURE 4 Dependence of $(k_{\alpha_2M}/k_{\alpha_1PI})^{app}$ on initial enzyme concentration. $[\alpha_1PI]_0 : [\alpha_2M]_0 = 8.7$; $[\alpha_1PI]_0 = 9.8$ μM. Calculations based on Equation (2).

The complexity of the system (the time-dependence of the concentration terms in Equation (10) does not allow quantitative analysis. Estimation of K_d was nevertheless attempted as follows, by using data obtained at $[\alpha_1PI]_0 \gg [E]_0$ and Equation (11), which was likely to substitute for Equation (10) under these conditions:

$$k'_{\alpha_2M} \approx \frac{k_{-1}k_{\alpha_2M}}{k_1[\alpha_1PI]_0}. \tag{11}$$

$$K_d^{app} \approx k'_{\alpha_2M}[\alpha_1PI]_0/k_{\alpha_2M}. \tag{11a}$$

The data points in Figure 3 for the highest three α_1PI concentrations (16–20 μM) were used to calculate k'_{α_2M} ($\approx k_{\alpha_1PI} \times (k_{\alpha_2M}/k_{\alpha_1PI})^{app}$; $k_{\alpha_1PI} = 2.7 \times 10^5\ M^{-1}\ s^{-1}$, this study). K_d^{app} was in turn calculated from Equation (11a), using $k_{\alpha_2M} = 2.1 \times 10^7\ M^{-1}\ s^{-1}$. A double reciprocal plot (not shown) of K_d^{app} vs $[\alpha_1PI]_0$ yielded a limiting K_d value of 2.1 ± 0.3 μM.

The Value of K_d as Determined by Gel Permeation

The titration with trypsin of the Sephadex G200 supernatant of a sample containing 2.5 μM each of α_2M and α_1PI is shown in Figure 5. The equivalence point was reached at 0.9 ± 0.02 μM trypsin, corresponding to a total inhibitor concentration of 2.3 ± 0.05 μM in the supernatant (or v_{outer}). Subtraction of $[\alpha_2M]_{outer} = 1.3 \pm 0.03$ μM (obtained from experiments with samples containing only α_2M) gave $[\alpha_1PI]_{outer} = 1.0 \pm 0.06$ μM. With $[\alpha_1PI]_{total} = 0.8 \pm 0.02$ μM (obtained from analysis of samples containing only α_1PI) $[\alpha_1PI]_{inner}$ was calculated to be 0.5 ± 0.13 μM. Substitution of these values into Equation (12) yielded $K_d = 0.8 \pm 0.32$ μM, in reasonable agreement with the kinetic estimate above.

$$K_d = \frac{[\alpha_1PI]_{inner}([\alpha_2M]_{outer} - ([\alpha_1PI]_{outer} - [\alpha_1PI]_{inner}))}{[\alpha_1PI]_{outer} - [\alpha_1PI]_{inner}}. \tag{12}$$

In Vivo Implications

Given the plasma concentrations of α_2M and α_1PI (2 ± 0.4 and 36 ± 12 μM, respectively[24]) the K_d estimate implies that >90% of α_2M in circulation should be in the complexed, protease-inactive state. The applicability of the model was tested in plasma. Titration of a fixed aliquot of plasma with trypsin showed a similar dependence of $(k_{\alpha_2M}/k_{\alpha_1PI})^{app}$ on enzyme concentration (Figure 6) and suggested that the α_2M–α_1PI complex must be

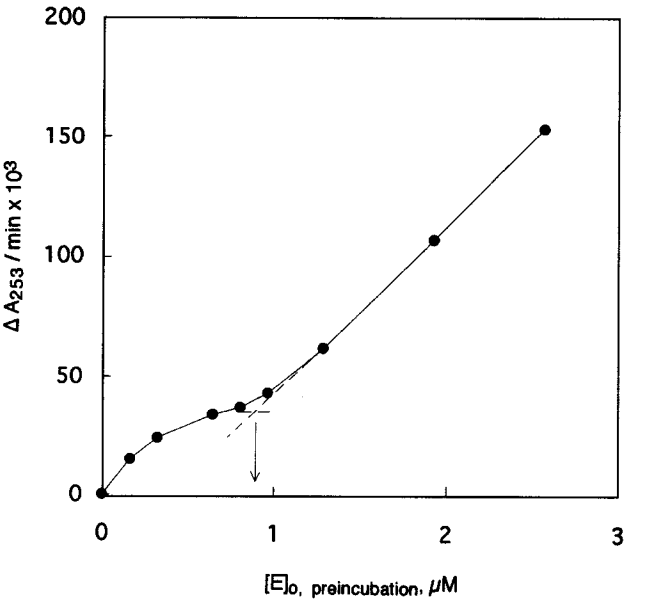


FIGURE 5 Titration with trypsin of the Sephadex G200 supernatent, following equillibration with a mixture of α_2M and α_1PI. Aliquots (80-μl) of the supernatent were preincubated with increasing amounts of enzyme in a total volume of 200 μl. The mixture was diluted 11-fold into substrate solution and residual esterase activity was determined as described in Methods. The total concentration of α_2M and α_1PI in the preincubation mixture is given by the point of intersectoin of the extension of the linear phase of the experimental curve with the horizontal (dashed) line marking the level of α_2M calculated from $[\alpha_2M]_{outer}$ (see Text).

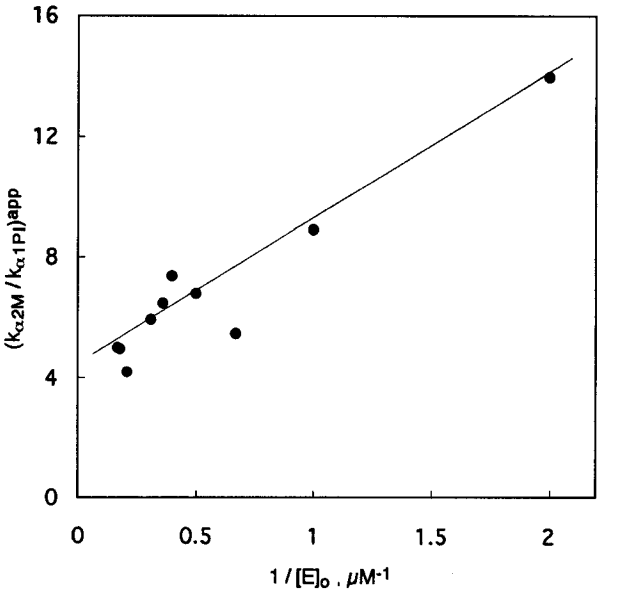


FIGURE 6 The variation of $(k_{\alpha_2M}/k_{\alpha_1PI})^{app}$ in the titration of a fixed aliquot of plasma with trypsin. $[\alpha_1PI]_0 : [\alpha_2M]_0 = 13$; $[\alpha_1PI]_0 = 15\,\mu M$. Calculations based on Equation (2).

relevant *in vivo*. The somewhat lower ordinate values (as compared to those in Figure 4) were presumably due to the contribution of inter-α-trypsin inhibitor to the inhibition of esteratic activity: $k_{\alpha_1 PI}[\alpha_1 PI]_{plasma}$ is of the same order of magnitude as $k_{I\alpha_1}[I\alpha]_{plasma}$.[25]

One obvious implication of the proposed interaction between α_2M and α_1PI is that the protease inhibitory role of the former in human circulation has been overestimated. What impact, if any, it has on the non-protease-inhibitory properties[26–29] of α_2M (involvement in growth factor regulation; affinity for various physiological ligands) is a further point of interest.

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