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Reaction of Proteinases with α_2-Macroglobulin: Evidence for Alternate Reaction Pathways in the Inhibition of Trypsin†

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ABSTRACT: Titration experiments were employed to measure the binding stoichiometry of α_2M for trypsin at high and low concentrations of reactants. These titration experiments were performed by measuring the SBTI-resistant trypsin activity and by direct binding measurements using ^{125}I-labeled trypsin. The binding stoichiometry displayed a marked dependence upon protein concentration. At high α_2M concentrations (micromolar), 2 mol of trypsin are bound/mol of inhibitor. However, at low α_2M concentrations (e.g., 0.5 nM), only 1.3 mol of trypsin were bound/mol of inhibitor. Sequential additions of subsaturating amounts of trypsin to a single aliquot of α_2M also resulted in a reduction in the final binding ratio. A model has been formulated to account for these observations. A key element of this model is the observation that purified 1:1 α_2M-proteinase complexes are not capable of binding a full mole of additional proteinase [Strickland et al. (1988) *Biochemistry 27*, 1458-1466]. The model predicts that once the 1:1 α_2M-proteinase complex forms, this species undergoes a time-dependent conformational rearrangement to yield a complex with greatly reduced proteinase binding ability. According to this model, the ability of α_2M to bind 2 mol of proteinase depends upon the association rate of the second enzyme molecule with the binary (1:1) complex, the enzyme concentration, and the rate of the conformational alteration that occurs once the initial complex forms. Modeling experiments suggest that the magnitude of the rate constant for this conformational change is in the order of 1-2 s^{-1}.

α_2-Macroglobulin (α_2M)[1] is a large (M_r = 718 000) plasma proteinase inhibitor that contains four identical subunits and has the ability to inhibit proteinases from all subclasses (Jones et al., 1972; Barrett & Starkey, 1973; Hall & Roberts, 1978; Sottrup-Jensen et al., 1984). This molecule is a member of a class of proteins that include pregnancy zone protein (PZP) and complement components C3 and C4. In addition to regions of conserved sequence among these proteins, all of them contain one or more internal β-cysteinyl-γ-glutamyl thiolester

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[1] Abbreviations: α_2M, α_2-macroglobulin; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PZP, pregnancy zone protein; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulfate; S-2222, *N*-benzoyl-L-isoleucyl-L-glutamylglycyl-L-arginine *p*-nitroanilide; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; TNS, 6-(*p*-toluidino)-2-naphthalenesulfonic acid.

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bonds (Sottrup-Jensen, 1980; Cambell, 1981; Howard, 1981; Thomas, 1982). This bond is cleaved upon reaction of these proteins with proteinases, which enables the activated form of these molecules to participate in covalent binding reactions. Certain primary amines are also capable of reacting with these molecules (Steinbuch et al., 1968; Barrett et al., 1979) by a nucleophilic cleavage of the thiolester bonds (Isenman & Kells, 1982; Isenman et al., 1981; Larsson & Björk, 1984; Strickland & Bhattacharya, 1984). In the case of α_2M, once the thiolester bonds are cleaved, the inhibitor undergoes a complex sequence of reactions (Strickland & Bhattacharya, 1984; Larsson et al., 1985) leading to a conformational change similar in several properties to that induced by proteinases (Barrett et al., 1979; Björk & Fish, 1982; Gonias et al., 1982; Larsson et al., 1987).

The inhibition of a proteinase is initiated by proteolysis of α_2M at a specific location of the polypeptide chain, termed the "bait" region (Harpel, 1973; Barrett et al., 1979; Swenson & Howard, 1979; Sottrup-Jensen et al., 1981). This cleavage gives rise to conformational changes in α_2M (Barrett et al., 1979; Gonias et al., 1982; Branegård et al., 1982) that result in inhibition of proteinase activity which can be measured by a reduction in activity toward large macromolecular substrates (Harpel, 1973; Barrett & Starkey, 1973; Swenson & Howard, 1979; Dangott et al., 1983) as well as the generation of receptor binding regions in the molecule (Van Leuven et al., 1986). The specific changes occurring in α_2M following proteolysis that lead to inhibition of proteinase activity and to the generation of receptor binding regions remain undefined.

One of the poorly understood aspects of the reaction of proteinases with α_2M is the observation that the binding of proteinases to the inhibitor occurs with varied stoichiometry and seems to depend on both the particular proteinase and the reactions conditions (Christensen & Sottrup-Jensen, 1984). A binding stoichiometry of 2 mol of proteinase/molecule of inhibitor has been reported for most small enzymes such as trypsin and chymotrypsin (Ganrot et al., 1966; Barrett et al., 1979; Björk et al., 1984). With other enzymes, such as thrombin and plasmin, a binding ratio of 1:1 to 1.3:1 is observed (Howell et al., 1983; Straight & McKee, 1984; Steiner et al., 1985, 1987). The final stoichiometry does not appear to be strictly related to size, since brinase, with a molecular weight close to that of thrombin, binds to α_2M with a stoichiometry of 1.9:1 (Larsson et al., 1988a). Further, fibroblast collagenase (52 kDa), which has been reported to react rapidly with α_2M ($k_1 > 10^6$ M^{-1} s^{-1}), binds to α_2M with a stoichiometry of 2:1 (Sottrup-Jensen & Birkedal-Hansen, 1989). Thus, a correlation between the rate at which a proteinase is inhibited and its binding stoichiometry has been noted (Howell, 1983; Straight & McKee, 1984), leading to the proposal that the association rate of a proteinase with α_2M is important in determining the binding ratio (Howell, 1983; Salvesen et al., 1983).

A key to understanding the reaction mechanism of a proteinase with α_2M is an appreciation of the relationship between the association rate of an enzyme with α_2M and its binding stoichiometry. The purpose of the present study was to address this issue, and it is demonstrated in this investigation that the final binding ratio between α_2M and trypsin can be altered simply by changing the reaction conditions. As the concentrations of the reactants are decreased from the micromolar range to the nanomolar level, a decrease in the maximal binding ratio from 2:1 to 1.3:1 occurs. On the basis of these and other observations a simple model for the reaction between α_2M and proteinases is described that establishes for the first time a relationship between inhibition rate and final binding stoichiometry.

Materials and Methods

α_2M was isolated from human plasma according to the method of Imber and Pizzo (1981) followed by immunoaffinity chromatography as described by Strickland et al. (1988). Trypsin from bovine pancreas was obtained from Sigma Chemical Co., St. Louis, MO. The active-site concentration was determined by titration with 4-nitrophenyl 4-guanidinobenzoate (NBGB, Merck, Darmstadt, West Germany; Chase & Shaw, 1970). Plasmin was prepared as described (Strickland et al., 1982), and the active-site concentration was determined by titration with NPGB. α-Chymotrypsin (Worthington) was prepared as a stock solution in 1 mM HCl, and the active-site concentration was determined by titration with *p*-nitrophenyl acetate as described by Bender et al. (1966). All of the proteinase concentrations used in the present study refer to the concentrations determined from active-site titrations. Hepes, SBTI, and TLCK were purchased from Sigma, whereas S-2222 was from KabiVitrum (Stockholm, Sweden).

The binding of trypsin to α_2M was studied by two different methods. The first method that was utilized is based on the observation (Ganrot, 1966) that α_2M is capable of protecting the bound enzyme from inactivation by SBTI. In this assay, aliquots of α_2M, at a final concentration of either 0.68 μM or 6.8 nM, were dispensed into several polypropylene tubes that were then incubated with increasing amounts of trypsin for 5 min in the case of high trypsin concentrations and 45 min in the case of low trypsin concentrations. Incubation times at low trypsin concentrations were chosen on the basis of a calculation of the measured rate constants, and sufficient time was allowed for 10–15 half-lives. Control experiments confirmed that the reagents are all stable for this time period. After incubation, a 10-fold molar excess of SBTI over trypsin was added. Following a 1-min incubation, remaining trypsin activity was measured at 410 nm with the chromogenic tripeptide substrate S-2222 in a Perkin-Elmer Lambda 5 spectrophotometer (Überlingen, West Germany). The buffer used in this assay was 50 mM Hepes and 0.15 M NaCl, pH 8.0. In control experiments, the specific activity of trypsin (measured by its ability to catalyze the cleavage of S-2222) was measured following each dilution to ensure that no loss of trypsin occurred by adsorption to surfaces. In a slightly different version of this assay, sequential additions (20 μL) of trypsin were made to one single batch of α_2M (final concentration, 1.1 μM) in a total volume of 2 mL. After intervals of either 10 or 30 min, 20 μL of the reaction mixture was removed and incubated with SBTI and S-2222 as described above, while a new addition of 20 μL of trypsin was made to the α_2M–trypsin reaction mixture. In this latter assay, corrections were made for the fact that the α_2M concentration during the experiment decreased slightly from 1.1 to 0.93 μM.

A second method was also employed to determine the amount of proteinase binding to α_2M by using ^{125}I-labeled trypsin. Trypsin was labeled with ^{125}I by using the Enzymobead reagent (Bio-Rad, Richmond, CA) to a specific activity of $\simeq$150 000 cpm/μg of α_2M, at final concentrations of either 0.85 μM or 0.5 nM, and was reacted with increasing amounts of ^{125}I-labeled trypsin for 5 min in the case of high α_2M concentration and for 40 min in the case of low α_2M concentration. The reaction was stopped by the addition of a 10-fold molar excess of SBTI over ^{125}I-labeled trypsin. The α_2M–^{125}I-labeled trypsin complexes were then isolated by immunoaffinity chromatography using the immobilized monoclonal antibody 7H11D6 (Strickland et al., 1988), and the

amount of bound trypsin was measured. No difference in the break point was observed when 0.02% Tween 20 was added to the buffer to minimize adsorption.

Gradient gel electrophoresis of α_2M–trypsin complexes was performed on 8–25% SDS–polyacrylamide gels (Pharmacia Phast System, Uppsala, Sweden). α_2M, at a final concentration of 2.2 μM, was reacted with trypsin at different molar ratios. After 45 min, an additional 0.2 mol of trypsin was added to some samples. After a further 45-min incubation, TLCK was added in a 10-fold excess over trypsin, and the sample was incubated for another 10 min. Samples of $\simeq$1 μg/well were analyzed under reducing conditions.

Protein concentrations were obtained by absorption measurements at 280 nm. The values for $E_{1\%}^{280nm}$ and molecular weights, respectively, used in the calculations were as follows: α_2M, 8.93 and 718 000 (Jones et al., 1972: Hall & Roberts, 1978); trypsin, 15.4 and 23 300 (Robinson et al., 1971; Walsh & Neurath, 1964); SBTI, 10.1 and 20 100 (Yamamoto & Ikenkar, 1967; Koide & Ikenakar, 1973); monoclonal antibody 7H11D6 belonging to subclass IgG_1, 14.0 and 160 000. The buffer used throughout this investigation was 50 mM Hepes and 0.15 M NaCl, pH 8.0.

The amount of α_2M–proteinase complex that was produced during the reaction of α_2M with proteinases was determined by using a sandwich ELISA. Briefly, wells of microtiter plates (Dynatech, Immulon II) were coated with 500 ng of monoclonal antibody 7H11D6. Dilutions of the samples containing the α_2M–proteinase mixture and standards were added to the wells. Following incubation and washing, the wells were incubated with a 1/1000 dilution of rabbit anti-human α_2M (Boehringer Mannheim). The amount of rabbit IgG bound was then detected by using a 1/1000 dilution of anti-rabbit IgG–alkaline phosphatase conjugate (Sigma).

Results

Effect of Reactant Concentration on Binding Stoichiometry of Trypsin with α_2M. A number of papers have documented that α_2M binds between 1 and 2 mol of proteinase, depending upon the specific enzyme studied. It has been observed that the binding stoichiometry of a proteinase for α_2M is related to the rate at which the proteinase reacts with this inhibitor (Howell et al., 1983; Straight & McKee, 1984), and as the association rate increases, the binding stoichiometry approaches 2.0 mol of proteinase bound/mol of inhibitor. At this time, the relationship between the association rate of an enzyme with α_2M and its stoichiometry is not well understood. It has been suggested (Salvesen et al., 1983; Howell et al., 1984) that once an enzyme cleaves some of the α_2M subunits, the inhibitor undergoes a rapid conformational change that prevents further proteinase binding. If this is the case, then the final binding stoichiometry of an enzyme for α_2M (when [proteinase] $\geq$ 2[α_2M]) will depend upon the concentration of the reactants and should therefore be reduced as the reactant concentrations are reduced.

To examine this hypothesis in more detail, the effect of reactant concentration on the binding ratio of trypsin and α_2M was studied. Trypsin was chosen as a model proteinase since it reacts rapidly with α_2M and binds at a mole ratio of 2:1 in the micromolar concentration range. The binding ratio was measured at different concentrations of reactants by titrating a fixed amount of immunoaffinity purified native α_2M with increasing concentrations of trypsin. Two different methods were employed to measure the amount of trypsin bound. The first (Figure 1) is based on the observation of Ganrot (1966) which measures the amount of trypsin activity present in the complex that is resistant to SBTI. At high concentrations of

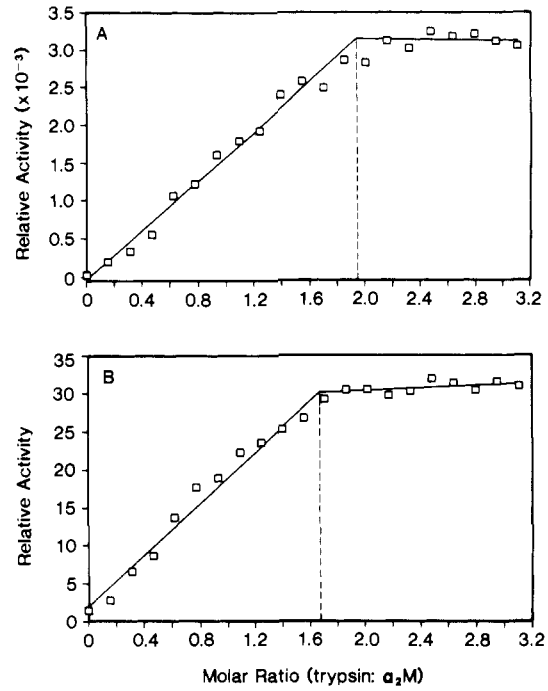


FIGURE 1: Stoichiometry of trypsin inhibition by α_2M analyzed by measurement of SBTI-resistant trypsin activity using the tripeptide substrate S-2222. (A) α_2M (0.68 μM) was incubated for 5 min with increasing mole ratios of trypsin. Free trypsin activity was inhibited by using a 10-fold excess of SBTI. (B) Concentration of α_2M was 6.8 nM. For these experiments, the incubation time was extended to 45 min. The lines are the best fit using the initial and final data points in the titration.

α_2M (0.68 μM) 1.95 mol of trypsin were bound/mol of α_2M (Figure 1A), which is consistent with previous papers (Ganrot, 1966; Barrett et al., 1979; Björk et al., 1984). However, when the concentration of α_2M was reduced to 6.8 nM, only 1.65 mol of trypsin were bound/mol of inhibitor (Figure 1B). The incubation time was extended to 45 min in these studies to allow sufficient time for completion of the reaction. It is unlikely that the reduced binding ratio observed at lower concentrations of trypsin arises from instability or adsorption of the trypsin, since measurement of trypsin amidolytic activity at 1 μM as well as 1 nM trypsin in 50 mM Hepes and 0.1 M NaCl, pH 8.0, revealed that very little trypsin activity was lost after 45 min of incubation, in agreement with the observations of Wang and Carpenter (1967). Further, measurement of the hydrolysis of S-2222 confirmed that no adsorption of trypsin to the polypropylene tubes was detected when the enzyme was diluted to a final concentration of 1 nM.

The results obtained by evaluating SBTI-resistant activity were confirmed by directly measuring the binding of trypsin to α_2M using ^{125}I-labeled trypsin. In these experiments, radiolabeled α_2M–trypsin complexes were separated from free ^{125}I-labeled trypsin by immunoaffinity chromatography as previously described (Strickland et al., 1988). A binding ratio between trypsin and α_2M of 2:1 was observed at high α_2M concentrations (0.85 μM) (Figure 2A). However, when the concentration of α_2M was reduced to 0.5 nM, only 1.3 mol of trypsin were bound/mol of inhibitor (Figure 2B). These results clearly demonstrate that the final binding ratio between α_2M and trypsin is altered by reducing the reactant concentrations. Moreover, these observations most likely extend to other proteinases since Salvesen et al. (1983) reported that the binding ratio between human neutrophil elastase and cathepsin G and α_2M were both reduced from 2:1 when the concentrations of initial reactants were reduced.

Effect of Sequential Additions of Trypsin on the Final

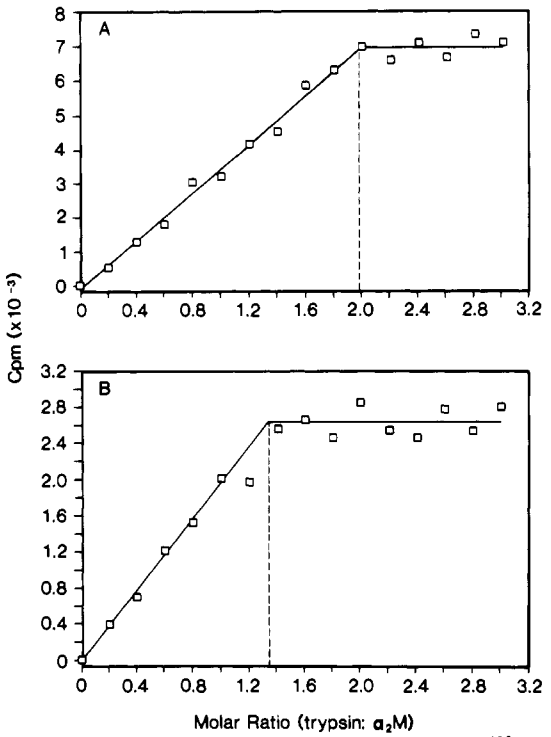


FIGURE 2: Stoichiometry of trypsin binding by α_2M using ^{125}I-labeled trypsin. (A) α_2M (0.85 μM) was incubated with ^{125}I-labeled trypsin for 5 min prior to removal of free trypsin by affinity chromatography over immobilized monoclonal antibody 7H11D6. (B) Trypsin concentration was 0.5 nM. The incubation time was extended to 45 min. The lines are the best fit using the initial and final data points in the titration.

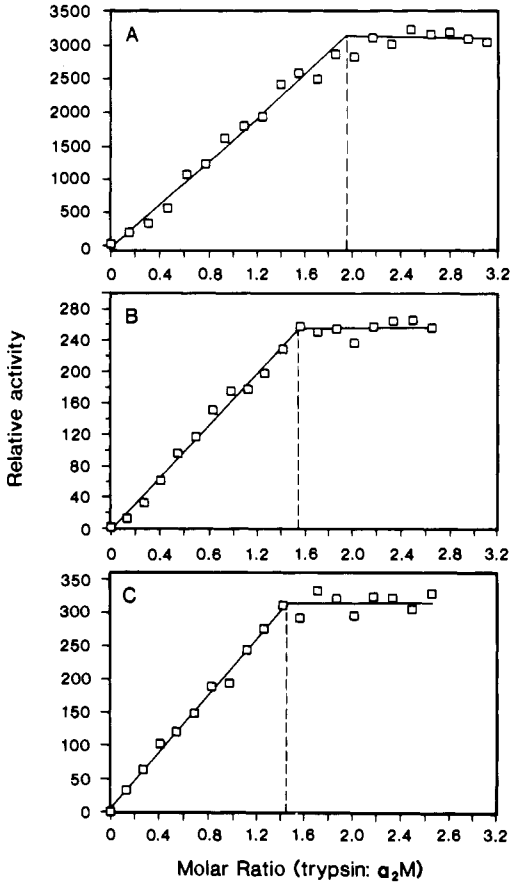


FIGURE 3: Effect of sequential addition of trypsin to α_2M on the final binding ratio determined by measurement of SBTI-resistant trypsin activity using the tripeptide substrate S-2222. (A) A fixed amount of α_2M (0.68 μM) was incubated with increasing concentrations of trypsin. Following incubation, excess trypsin was inhibited by addition of a 10-fold molar excess of SBTI. Trypsin activity was measured by using S-2222. (B) Sequential additions of trypsin to a single batch of α_2M. Following each addition of trypsin, the samples were incubated for 10 min, and an aliquot was removed, incubated with a 10-fold molar excess of SBTI, and assayed for trypsin activity. The initial α_2M concentration was 1.1 μM. The results were corrected for the slight dilution of α_2M that occurred. (C) Sequential additions as in (B) but with 30 min between additions. The lines are the best fit using the initial and final data points in the titration.

Stoichiometry between α_2M and Trypsin. Howell et al. (1983) have reported that serial additions of nonsaturating amounts of trypsin reduce the final stoichiometry somewhat. The following studies were designed to explore this in more detail. An aliquot of trypsin was added to α_2M and allowed to react for either 10 (Figure 3B) or 30 min (Figure 3C) prior to the next addition of trypsin. For comparison, the results obtained in a titration using a single addition of trypsin to aliquots of α_2M are shown (Figure 3A). The binding capacity of α_2M is reduced in those titrations in which sequential additions of trypsin were added to the inhibitor. Thus, 10-min incubations between each addition of trypsin reduce the amount of trypsin bound from 1.95 mol in the control to 1.65 mol of trypsin bound/mol of inhibitor. Extending the incubation time between each addition to 30 min reduces the amount of trypsin bound even further to a value of 1.45 mol of enzyme bound/mol of α_2M. Since 2 mol of trypsin can readily be incorporated into α_2M when an excess of the enzyme is reacted with the inhibitor at these concentrations, these data and those of the previous experiment are supportive of a branching pathway in which the initially formed α_2M–proteinase complex undergoes a time-dependent rearrangement that greatly reduces further proteinase binding.

Measurement of the Amount of Complex Produced during the Reaction of α_2M with Proteinases. Straight and McKee (1984) have proposed a mechanism for the reaction of proteinases with α_2M. In their mechanism, once a proteinase associates with the inhibitor and cleaves at the bait region of the first proteinase binding site, the proteinase can either dissociate or become irreversibly bound as a consequence of the "trapping" reaction. Dissociation of the proteinase from the complex following cleavage leads to "nonproductive" cleavage of α_2M in which α_2M subunits are cleaved but the enzyme is not incorporated into the complex. This model was proposed on the basis of the observation that a large number of enzymes (e.g., plasmin and thrombin) demonstrate this nonproductive cleavage. If this model is valid, then one would expect that the reaction of α_2M with subsaturating concentrations of enzyme would lead to the production of greater than stoichiometric amounts of conformationally altered α_2M. To measure this, α_2M was incubated with chymotrypsin or plasmin at various mole ratios and the amount of modified α_2M formed measured by a sandwich ELISA using monoclonal antibody 7H11D6, which is specific for these α_2M

Table I: Concentration of Modified α_2M Formed during the Reaction of Proteinases with the Inhibitor

[α_2M] (μM)	[enzyme] (μM)	mol of enzyme/ mol of α_2M	[modified α_2M]$_{formed}$ (μM)	[modified α_2M]$_{expected}$ (μM)
		Chymotrypsin		
1.32	1.32	1.0	1.19 ± 0.06	1.32
1.32	0.66	0.5	0.55 ± 0.66	0.66
1.32	0.13	0.1	0.16 ± 0.04	0.13
		Plasmin		
6.00	6.00	1.0	5.72 ± 0.51	6.00
6.00	3.00	0.5	3.04 ± 0.33	3.00
6.00	0.60	0.1	0.50 ± 0.06	0.60

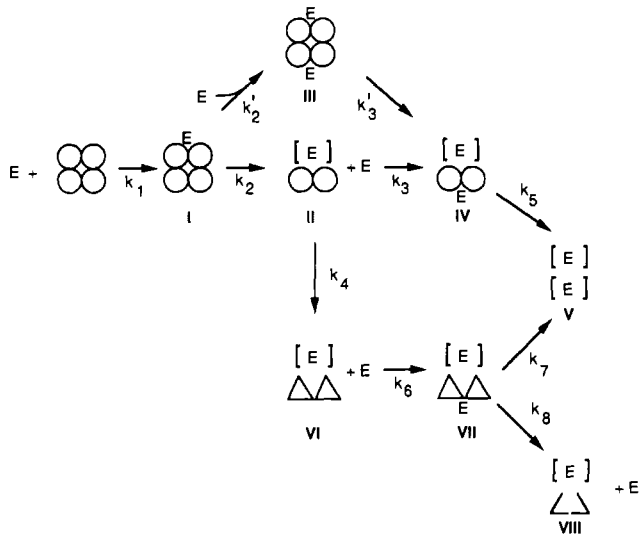


FIGURE 4: Model for the reaction of proteinases with α_2M. (E) proteinase; (I–VIII), different forms of reacted α_2M molecules in this model; (k_1–k_8), rate constants for the different steps in the reaction. In this model, the trapped proteinase is enclosed by brackets, while triangles are used to designate a conformational rearrangement that occurs with rate constant k_4. Nonproductive cleavage is denoted by the open triangles.

derivatives (Strickland et al., 1988). The results of this study are summarized in Table I, where it is apparent that the concentration of modified α_2M produced during the reaction is very close to that calculated by assuming 1 mol of proteinase reacts with 1 mol of inhibitor. Thus, nonproductive cleavage does not occur when the first proteinase associates with the inhibitor, and consequently, the trapping efficiency appears extremely high for the first mole of proteinase bound.

Description of a Model for the Reaction of Proteinases with α_2M. A simple model that relates binding stoichiometry to association rate and accounts for the concentration dependence of the binding stoichiometry is presented in Figure 4. In this model, a proteolytic enzyme (E) reacts with α_2M. Following proteolysis, conformational changes occur that lead to complex II (Figure 4) in which some of the α_2M subunits have been cleaved and the enzyme is "entrapped". At this point, two pathways exist for this complex. The first pathway is one in which this complex reacts with a second mole of proteinase to give complex IV, which then undergoes conformational changes that result in inhibition of proteinase activity with the formation of complex V. A complex with properties similar to complex V (i.e., 2 mol of proteinase bound/mol of α_2M, four thiolester bonds hydrolyzed, four α_2M subunits cleaved) is isolated when native α_2M, at a concentration of 1 μM, reacts with an excess (≥2 mol of trypsin/mol of inhibitor) of trypsin.

A second pathway is proposed in which complex II can undergo a conformational change prior to the binding of a second mole of proteinase to form complex VI (Figure 4). It is envisioned that this step involves additional conformational changes within the complex. Interestingly, the existence of complexes with properties similar to complex VI have been documented by isolating and characterizing binary α_2M–proteinase complexes (Strickland et al., 1988). These complexes, prepared by reacting large excesses of native α_2M with small amounts of proteinase, contain 1 mol of proteinase/mol of inhibitor. The complexes have between 2 and 3 α_2M subunits cleaved and two thiolester bonds hydrolyzed. Further, the magnitude of the conformational alterations is approximately half of that observed when α_2M is reacted with an excess (≥2 mol of trypsin/mol of α_2M) of trypsin. Despite the fact that these complexes contain alterations that appear to be restricted to one of two functional units in the inhibitor, binding studies reveal that they are somewhat limited in their ability to bind additional proteinase. However, the remaining two α_2M subunits are readily cleaved (Strickland et al., 1988) when incubated with an excess of trypsin. In Figure 4, the conversion of complex VI to complex VIII accounts for this nonproductive cleavage of α_2M, which has been observed in previous studies examining the reaction of immobilized trypsin with α_2M (Björk, 1984) and in studies analyzing the reaction of plasmin (Steiner et al., 1987; Pochon, 1987; Roche & Pizzo, 1987) or thrombin (Straight & McKee, 1984; Steiner et al., 1985) with α_2M. The majority of complex VI appears to be converted to complex VIII via this pathway; however, since a small amount of proteinase is incorporated into these complexes (Strickland et al., 1988), a pathway (complex VI to complex V) has been included in the model allowing for incorporation of additional proteinase into the binary complex.

The model also shows a second possible pathway for complex I, in which an enzyme can associate with this complex prior to conformational changes to form complex III. This complex then undergoes a series of conformational change to ultimately form complex V.

The relationship between binding stoichiometry and association rate is demonstrated by examination of the differential rate equations (see Appendix) for the scheme shown in Figure 4. The ratio of complex V to complex VIII at $t = \infty$ simplifies to

$$\frac{[V]_{t=\infty}}{[VIII]_{t=\infty}} = \frac{k_7}{k_8} + \left(1 + \frac{k_7}{k_8}\right)\left(\frac{k_3E}{k_4} + \frac{k_2'E}{k_2} + \frac{k_2'k_3E^2}{k_2k_4}\right)$$

and when $k_7 \approx k_2' \approx 0$

$$[V]_{t=\infty}/[VIII]_{t=\infty} = k_3E/k_4$$

Thus, in the simple situation when $k_7 \approx k_2' \approx 0$ and when $[E] \geq 2[\alpha_2M]$, the model predicts that ternary α_2M–proteinase complexes will be produced when $k_3[E] \gg k_4$. When $k_3[E] \ll k_4$, the model predicts that binary complexes will predominate, while when $k_3[E] \approx k_4$, both binary and ternary complexes will be produced.

Simulation of the Reaction of Proteinases with α_2M. Studies examining the reaction of several enzymes with α_2M have yielded rate constants for the association of trypsin (Christensen & Sottrup-Jensen, 1984), plasmin (Steiner et al., 1987; Christensen & Sottrup-Jensen, 1984), and thrombin (Steiner et al., 1985) with this inhibitor. These values for k_1 are summarized in Table II. To date, little information exists concerning the rate of association of the second mole of enzyme, and thus the value of k_2' was assumed to be close to that

Table II: Parameters Used To Simulate the Reaction of Trypsin, Plasmin, and Thrombin with α_2-Macroglobulin

parameter	trypsin	trypsin	plasmin	thrombin
[enzyme] (μM)	4.0	0.002	4.0	4.0
[α_2M] (μM)	0.2	0.0001	0.2	0.2
k_1 (M^{-1} s^{-1})	2.0×10^7 [a]	2.0×10^7 [a]	1.3×10^5 [b]	3.2×10.3 [c]
k_2 (s^{-1})	10	10	10	10
k_2' (M^{-1} s^{-1})	2.0×10^7	2.0×10^7	1.3×10^5	3.2×10^3
k_3 (M^{-1} s^{-1})	2.0×10^6 [a]	2.0×10^6 [a]	3.0×10^3 [a]	3.0×10^2 [d]
k_4 (s^{-1})	1	1	1	1
k_7 (s^{-1})	2	2	0	0
k_8 (s^{-1})	5	5	5	5
calcd binding ratio	2.0	1.3	1.1	1.0
obsd binding ratio	2.0	1.3	1.1–1.3	1.0

[a] From Christensen and Sottrup-Jensen (1984). [b] From Steiner et al. (1987). [c] From Steiner et al. (1985). [d] Estimated from data of Steiner et al. (1985).

for k_1. Christensen and Sottrup-Jensen (1984) measured the reaction of trypsin and plasmin with α_2M and found that the reaction of the second mole of enzyme with the inhibitor–proteinase complex is considerably slower than the reaction of the native inhibitor with the first enzyme molecule. This observation is supported by other studies (Steiner et al., 1985, 1987; Dangott et al., 1983). One possible interpretation of these data is that the slower reaction represents the association of a second mole of proteinase with the conformationally altered α_2M–proteinase complex, and thus their values have been utilized for estimates of k_3. Also summarized in Table II are the observed binding ratios for these three enzymes. On the basis of these values for k_1 and k_3 it is possible to estimate the magnitude of k_4 that results in a reduction in the amount of trypsin bound when the concentration of reactants is reduced in the experiments reported in Figures 1 and 2. A value between 1 and 2 s^{-1} allowed for an excellent correlation between the observed and calculated binding ratio for trypsin at the two concentrations employed as well as for the other two proteinases (Table II). While the values of k_7 and k_8 are somewhat arbitrary, the ratio of these rate constants was estimated from experiments examining the binding of enzyme to purified binary complexes (Strickland et al., 1988).

A simulation of the reaction of α_2M with trypsin at high concentration of reactants (1 μM) demonstrated that only a single species, complex V (with 2 mol of enzyme bound/mol of inhibitor), exists in significant amounts. However, when the reactant concentrations are reduced to the nanomolar range, considerable amounts of complex VI form, which is converted to complex VIII containing 1 mol of enzyme bound/mol of inhibitor. This gives the final binding stoichiometry of 1.3 mol of trypsin bound/mol of inhibitor.

Additional simulation experiments revealed that the reaction of thrombin with α_2M, using the rate constants in Table II, generates complex VIII, with only trace amounts of complex V formed. In the case of plasmin, both complexes V and VIII form, predicting the existence of both 1:1 and 2:1 α_2M–proteinase complexes. Substantial evidence indicates that despite the large size of plasmin, some 2:1 α_2M–plasmin complexes do in fact form. This has been demonstrated by titration studies (Steiner et al., 1987) as well as by visualization of the α_2M–plasmin complex by electron microscopy using a gold-labeled monoclonal antibody to the heavy chain of plasmin (Gonias et al., 1988).

Discussion

One of the puzzling aspects of α_2M chemistry is the observation that the binding stoichiometry depends not only upon the particular proteinase but, as demonstrated in the present study, also upon the reaction conditions. Thus, it is possible to manipulate the amount of trypsin bound to α_2M simply by altering the reactant concentrations, and the results of the present investigation document that the stoichiometry is decreased as the concentrations of α_2M and trypsin are reduced. A clear comprehension of these observations is important in understanding the inhibition mechanism, and any scheme describing the reaction pathways of proteinases with α_2M must account for this observation. The scheme presented in Figure 4 represents a simple model describing pathways available for the reaction of a proteinase with α_2M and adequately accounts for the varied stoichiometry reported for different enzymes and for the lowered stoichiometry observed when the reactant concentrations are reduced. The model predicts that the ability of α_2M to bind two proteinase molecules depends upon the association rate of the second enzyme molecule with the binary α_2M–proteinase complex, as well as the proteinase concentration, and the rate at which the binary α_2M–proteinase complex undergoes conformational alterations to produce a complex with reduced proteinase binding capacity (complex VI).

The existence of these complexes (complex VI) has been documented by preparing and characterizing binary α_2M–proteinase complexes (Strickland et al., 1988). When compared with ternary α_2M–proteinase complexes (2 mol enzyme bound/mol of α_2M), the change in migration mobility upon native PAGE, reflective of hydrodynamic changes, was intermediate between that of native α_2M and that of ternary α_2M–proteinase complexes. Likewise, the enhancement of TNS fluorescence that occurs when a proteinase reacts with α_2M (Strickland & Bhattacharya, 1984) was intermediate between that of native α_2M and that of the ternary α_2M–proteinase complex. Despite the fact that the conformational changes appear to primarily alter one of the two functional units within the molecule, the complexes were limited in their ability to bind additional proteinase. Similar results have been observed by Pochon (1987), who examined the binding of chymotrypsin to 1:1 α_2M–plasmin complexes. His observations indicated that only 50% of the total chymotrypsin that was bound to the 1:1 complex remained associated as a ternary complex upon gel filtration.

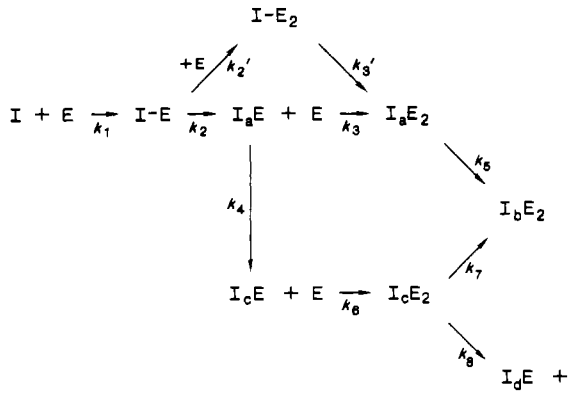
A key question that remains to be addressed is the nature of the changes that occur in the initial α_2M binary complex (complex II, Figure 4) that produce a complex (complex VI, Figure 4) with reduced binding capacity. Pochon (1987) has suggested that the degree of covalent binding might influence the incorporation of chymotrypsin into the 1:1 α_2M–plasmin complex, and he observed much less incorporation of chymotrypsin into complexes in which the plasmin was covalently bound to α_2M. Christensen and Sottrup-Jensen (1984) have proposed that the conformational change might affect the accessibility of the remaining bait regions, possibly due to a change in subunit interactions. Altered subunit interactions following proteolysis of α_2M are well established. Studies investigating the properties of purified α_2M subunits prepared by reduction with thioredoxin (Larsson et al., 1988b) confirm that the subunits reassociate into a tetramer following reaction with proteinases or with methylamine. Likewise, studies investigating the properties of α_2M "half-molecules" prepared by reduction with dithiothreitol indicate that after reaction with trypsin or methylamine, the half-molecule reassociates to form a tetramer (Gonias & Pizzo, 1983). The exact regions within the subunits that are responsible for these changes and the impact the changes might have an proteinase binding remain to be identified.

The model in Figure 4 also accounts for nonproductive cleavage which is well documented in the literature. Studies measuring the amount of α_2M–proteinase complex formed indicate that the inhibition of the first enzyme by α_2M is extremely efficient, and it appears that nonproductive cleavage primarily occurs when the second mole of enzyme reacts with the 1:1 complex. It should be emphasized that the reaction of proteinases with α_2M is extremely complex, and the simple scheme in Figure 4 most likely does not contain all of the intermediate steps in the process. Stopped-flow kinetic data for the reaction of trypsin (Dangott et al., 1983) and chymotrypsin (Favaudon et al., 1988) with the inhibitor have been reported. In the latter paper, it was observed that the data could be fit to a triexponential rate equation, which each phase appearing to be first order. While the relationship between these phases and events that occur within the α_2M molecule remain to be established, it does appear that this approach will

yield further insight into the reaction mechanism.

Appendix: Solution of the Differential Rate Equations

The integrated rate equations are derived for the mechanism



where I is α_2M; E is enzyme; I–E, I_aE, I_cE, and I_dE denote different conformational states of α_2M with 1 mol of enzyme bound; I–E_2, I_aE_2, I_bE_2, and I_cE_2 denote different conformational states of α_2M with 2 mol of enzyme bound; and k_1, k_2, ..., k_8 represent the rate constant for each step.

Let $x = [I]$, $y = [I–E]$, $z = [I–E_2]$, $u = [I_aE]$, $v = [I_aE_2]$, $w = [I_cE]$, $f = [I_cE_2]$, $g = [I_dE]$, $h = [I_bE_2]$, $E = [E]$, and t = time. The differential rate equations for each species are

$$dx/dt = -k_1Ex \tag{A1}$$

$$dy/dt = -(k_2 + k_2'E)y + k_1Ex \tag{A2}$$

$$dz/dt = -k_3'z + k_2'Ey \tag{A3}$$

$$du/dt = -(k_3E + k_4)u + k_2y \tag{A4}$$

$$dv/dt = -k_5v + k_3'z + k_3Eu \tag{A5}$$

$$dw/dt = -k_6Ew + k_4u \tag{A6}$$

$$df/dt = -(k_7 + k_8)f + k_6Ew \tag{A7}$$

$$dg/dt = k_8f \tag{A8}$$

$$dh/dt = k_5v + k_7f \tag{A9}$$

The initial conditions are $x(t = 0) = x_0$; all others are zero at $t = 0$.

Let

$$A_1 = k_1E,\ A_2' = k_2'E, \text{ etc.} \tag{A10}$$

$$B = k_2 + A_2' \tag{A11}$$

$$C = A_3 + k_4 \tag{A12}$$

$$D = k_7 + k_8 \tag{A13}$$

From (A1) we obtain

$$x(t) = x_0e^{-A_1t} \tag{A14}$$

Substituting this into (A2) yields

$$y(t) = \frac{A_1x_0}{B - A_1}(e^{-A_1t} - e^{-Bt}) \tag{A15}$$

Continuing in this manner, one successively solves (A3), (A4), ..., (A9), eventually obtaining for $g(t)$ and $h(t)$ the expression

$$g(t) = A_1k_2k_4A_6k_8x_0 \times \left\{ \frac{\frac{1}{A_1}(1 - e^{-A_1t}) - \frac{1}{D}(1 - e^{-Dt})}{(B - A_1)(C - A_1)(A_6 - A_1)(D - A_1)} - \frac{\frac{1}{B}(1 - e^{-Bt}) - \frac{1}{D}(1 - e^{-Dt})}{(B - A_1)(C - B)(A_6 - B)(D - B)} + \frac{\frac{1}{C}(1 - e^{-Ct}) - \frac{1}{D}(1 - e^{-Dt})}{(C - B)(C - A_1)(A_6 - C)(D - C)} - G\left[\frac{\frac{1}{A_6}(1 - e^{-A_6t}) - \frac{1}{D}(1 - e^{-Dt})}{(B - A_1)(D - A_6)}\right]\right\} \tag{A16}$$

where

$$G = \frac{1}{(C - A_1)(A_6 - A_1)} - \frac{1}{(C - B)(A_6 - B)} + \frac{B - A_1}{(C - B)(C - A_1)(A_6 - C)} \tag{A17}$$

$$h(t) = \frac{k_7}{k_8}g(t) + \frac{A_1k_5x_0H(A_1)}{(B - A_1)(k_5 - A_1)}\left\{\frac{1}{A_1}(1 - e^{-A_1t}) - \frac{1}{k_5}(1 - e^{-k_5t})\right\} - \frac{A_1k_5x_0H(B)}{(B - A_1)(k_5 - B)}\left\{\frac{1}{B}(1 - e^{-Bt}) - \frac{1}{k_5}(1 - e^{-k_5t})\right\} + \frac{A_1A_2'k_3'k_5x_0}{(k_3' - B)(k_3' - A_1)(k_5 - k_3')}\left\{\frac{1}{k_3'}(1 - e^{-k_3't}) - \frac{1}{k_5}(1 - e^{-k_5t})\right\} + \frac{A_1k_2A_3k_5x_0}{(C - B)(C - A_1)(k_5 - C)} \times \left\{\frac{1}{C}(1 - e^{-Ct}) - \frac{1}{k_5}(1 - e^{-k_5t})\right\} \tag{A18}$$

where

$$H(p) = \frac{A_2'k_3'}{k_3' - p} + \frac{k_2A_3}{C - p} \tag{A19}$$

As $t \to \infty$, these reduce to

$$g(\infty) = k_2k_4k_8x_0/BCD \tag{A20}$$

and

$$h(\infty) = (k_7/k_8)g(\infty) + (x_0/BC)(CA_2' + k_2A_3) \tag{A21}$$

Thus (A20) and (A21) yield, upon recalling (A10)–(A13)

$$\frac{h(\infty)}{g(\infty)} = \frac{k_7}{k_8} + \left(1 + \frac{k_7}{k_8}\right)\left(\frac{k_3E}{k_4} + \frac{k_2'E}{k_2} + \frac{k_2'k_3E^2}{k_2k_4}\right) \tag{A22}$$

If one now assumes that k_7 and k_2' are negligible, i.e., $k_7 \sim 0$ and $k_2' \sim 0$, then (A22) becomes

$$h(\infty)/g(\infty) \approx k_3E/k_4 \tag{A23}$$

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