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Functional modifications of α_2-macroglobulin by primary amines

Kinetics of inactivation of α_2-macroglobulin by methylamine, and formation of anomalous complexes with trypsin

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The unique steric inhibition of endopeptidases by human α_2M (α_2-macroglobulin) and the inactivation of the latter by methylamine were examined in relation to each other. Progressive binding of trypsin by α_2M was closely correlated with the loss of the methylamine-reactive sites in α_2M: for each trypsin molecule bound, two such sites were inactivated. The results further showed that, even at low proteinase/α_2M ratios, no unaccounted loss of trypsin-binding capacity occurred. As α_2M is bivalent for trypsin binding and no trypsin bound to electrophoretic slow-form α_2M was observed, this indicates that the two sites must react (bind trypsin) in rapid succession. Reaction of [^{14}C]methylamine with α_2M was biphasic in time; in the initial rapid phase complex-formation with trypsin caused a largely increased incorporation of methylamine. In the subsequent slow phase trypsin had no such effect. These results prompted further studies on the kinetics of methylamine inactivation of α_2M with time of methylamine treatment. It was found that conformational change of α_2M and decrease in trypsin binding (activity resistant to soya-bean trypsin inhibitor) showed different kinetics. The latter decreased rapidly, following pseudo-first-order kinetics. Conformational change was much slower and followed complex kinetics. On the other hand, binding of ^{125}I-labelled trypsin to α_2M did follow the same kinetics as the conformational change. This discrepancy between total binding (^{125}I radioactivity) and trypsin-inhibitor-resistant binding of trypsin indicated formation of anomalous complexes, in which trypsin could still be inhibited by soya-bean trypsin inhibitor. Further examination confirmed that these complexes were proteolytically active towards haemoglobin and bound ^{125}I-labelled soya-bean trypsin inhibitor to the active site of trypsin. The inhibition by soya-bean trypsin inhibitor was slowed down as compared with reaction with free trypsin. The results are discussed in relation to the subunit structure of α_2M and to the mechanism of formation of the complex.

α_2-Macroglobulin (α_2M) is unique among the proteinase inhibitors present in the circulation of mammals, by virtue of its typical steric mode of inhibition of nearly all known endopeptidases (Starkey & Barrett, 1977). Only enzymically active proteinases are bound, indicating that the proteolytic event (cleavage of the polypeptide chain of α_2M) (Harpel, 1977) triggers the actual formation of the α_2M–proteinase complex. The complex maintains the enzymic activity of the proteinase towards low-molecular-weight substrates, but proteolysis is inhibited almost completely. Analytically, the complexes are distinguished from native α_2M by their faster electrophoretic mobility despite a more alkaline pI (Ohlsson & Skude, 1976; Barrett *et al.*, 1979; Parsons & Romeo, 1980; Van Leuven *et al.*, 1981*a*). Of biological relevance was the finding of a cellular receptor specific for α_2M–proteinase complexes (Debanne *et al.*, 1975; Van Leuven *et al.*, 1979; Kaplan & Nielsen, 1979). Recognition leads to rapid internalization of α_2M–proteinase complexes by a receptor-mediated endocytosis mechanism (Van Leuven *et al.*, 1980), similar in characteristics to the one proposed for low-density

Abbreviation used: α_2M, α_2-macroglobulin.

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lipoprotein (Goldstein *et al.*, 1979). Rapid clearance of α_2M–proteinase complexes from the circulation *in vivo* (Ohlsson & Skude, 1976) is likely to be mediated by a similar mechanism.

Primary amines inactivate α_2M as a proteinase inhibitor (Steinbuch *et al.*, 1968), by a covalent modification of a 'pseudo-active centre' (Swenson & Howard, 1979). Treatment of α_2M with proteinase or with methylamine results in the same conformational form of α_2M, as judged from electrophoretic mobility, isoelectric-focusing pattern and kinetics of receptor-mediated endocytosis by fibroblasts in culture (Van Leuven *et al.*, 1981*a*). We have further shown that proteinases transiently activate the methylamine-reactive site, which was interpreted to show that the methylamine-reactive site actually holds α_2M in the native conformation and is activated by proteolysis leading to the formation of complex (Van Leuven *et al.*, 1981*b*).

The present work was undertaken to examine quantitatively the inter-relationship of trypsin and methylamine actions on α_2M. A direct correlation was found between the trypsin-binding sites and the 'methylamine-reactive' sites on the formation of α_2M–trypsin complex. Studies on the kinetics of methylamine incorporation were done in relation to conformational change and trypsin-binding capacity of α_2M. The results show that under certain conditions α_2M–trypsin complexes can be formed that are proteolytically active towards haemoglobin and that bind soya-bean trypsin inhibitor to the active site of trypsin in the complex.

Experimental

Materials

The Radiochemical Centre (Amersham, Bucks., U.K.) supplied us with [^{14}C]methylamine (specific radioactivity 61.1 mCi/mmol) and with Na^{125}I. 1 - Chloro - 4 - phenyl - 3 - tosylamidobutan - 2 - one ('TPCK')-treated trypsin and soya-bean trypsin inhibitor were from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.). Radioautography was done on ^{3}H-Ultrofilm (LKB Produkter, Stockholm, Sweden).

Purification of α_2M

Human citrated plasma (haptoglobin type 1–1) was obtained from the plasmapheresis service of the local blood bank and processed on the day of donation. In the initial stages of purification only plastic laboratory ware was used.

Plasma α_2M was isolated after treatment of the plasma with $BaCl_2$ and $BaSO_4$ as described previously (Van Leuven *et al.*, 1979). Further purification steps were: precipitation of α_2M with polyethylene glycol-6000 (precipitate between 4 and 8%, w/v) and gel filtration on Ultrogel AcA-22 (LKB) [column dimensions 5 cm × 95 cm; eluent phosphate-buffered saline (0.15 M-NaCl/20 mM-sodium phosphate buffer, pH 7.4)]. The final purification step consisted of chromatography on a column (2.6 cm × 90 cm) of Blue Sepharose (Pharmacia, Uppsala, Sweden), with 25 mM-sodium phosphate buffer, pH 7.7.

Quantitative determination of α_2M after each step and in column fractions was performed by 'rocket' immunoelectrophoresis (Van Leuven *et al.*, 1978). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. A typical purification procedure, starting from 300 ml of plasma, resulted in a preparation containing 97% α_2M in a yield of 58%.

Electrophoretic techniques in thin-layer polyacrylamide gels

Electrophoretic mobility of native proteins was examined in 5% polyacrylamide gels. A discontinuous buffer system was used. Reservoir buffer (upper and lower) was 41 mM-Tris/40 mM-borate buffer, pH 8.6; stacking gel was 4% polyacrylamide in 54 mM-Tris/20 mM-H_2SO_4 buffer, pH 6.1; separating gel was in 95 mM-Tris/HCl buffer, pH 5.7. Samples were prepared in reservoir buffer containing 10% (v/v) glycerol. Gels were cast in cassettes (Pharmacia) of dimensions 8 cm × 8 cm × 0.27 cm and run for 3 h at 125 V.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed as described by Laemmli (1970) on gradient gels (4–20% polyacrylamide), of dimensions 8 cm × 14 cm × 0.27 cm (Pharmacia). Samples were prepared in buffer containing 1% (w/v) sodium dodecyl sulphate and 1% (v/v) 2-mercaptoethanol and left for 10 min at 80°C before application on the gel. Electrophoresis was for 16 h at 50 V. The apparatus used was a Pharmacia GE-2/4 gel-electrophoresis tank with circulating buffer, operated at room temperature.

Isoelectric focusing was done on a Multiphor apparatus and the appropriate electrodes for focusing (LKB). Gels were commercially available and run as recommended by the manufacturer (PAG-PLATE, pH 3.5–9.5; LKB). All gels were fixed in 10% (w/v) trichloroacetic acid, stained with Coomassie Brilliant Blue (at 60°C) and destained by diffusion. After shrinking of the gels in methanol/water (1:1, v/v) overnight, the gels were dried for radioautography. Photographs were made with a Polaroid camera with positive/negative type 665 film.

Quantitative determination of radioactivity was done by excision of Coomassie Brilliant Blue-stained bands with a razor-blade. The pieces of gel were dissolved in 1 ml of concentrated H_2O_2 at 56°C overnight. After addition of 15 ml of scintillation 'cocktail' (Instagel II; Packard Instruments, La

Grange, IL, U.S.A.), radioactivity was counted (Packard Tri-Carb liquid-scintillation counter). Counting efficiency was determined with an internal standard.

Trypsin activity

Trypsin enzymic activity towards benzoylarginine *p*-nitroanilide was measured at 37°C as described previously (Van Leuven *et al.*, 1981*a*): the assay mixture contained 0.1 M-Tris/HCl buffer, pH 8.2, 20 mM-$CaCl_2$, 2 mM-benzoylarginine *p*-nitroanilide and enzyme in a final volume of 2.5 ml. Absorbance at 410 nm was continuously recorded. Enzymic activity towards Chromozym PL (tosylglycylprolyllysine *p*-nitroanilide; Boehringer, Mannheim, Germany) was measured under similar conditions, at a substrate concentration of 0.3 mM. Activities are expressed as the change in A_{410}/min at 37°C. When indicated, soya-bean trypsin inhibitor was added, equivalent to the amount of trypsin present on a weight basis.

Proteolytic activity was measured towards [^{3}H]-acetylated haemoglobin. Bovine haemoglobin (type I; Sigma Chemical Co., St. Louis, MO, U.S.A.) was labelled with [^{3}H]acetic anhydride (3 Ci/mmol; The Radiochemical Centre): 600 mg of haemoglobin was treated with 25 mCi of anhydride in 0.5 M-borate/1.4 M-sodium acetate buffer, pH 9.0, for 24 h at 0°C. The labelled protein was collected and freed from anhydride that had not reacted by repeated precipitation with trichloroacetic acid and finally dialysed against 0.16 M-NaCl. The final preparation contained 0.62 mg of protein/ml and was stored at −20°C. It contained 4500 c.p.m./μg of protein, of which 0.4% was not precipitated by trichloroacetic acid.

The assay mixture contained, in a final volume of 0.5 ml, 50 mM-Tris/HCl buffer, pH 8.2, 100 μl of the labelled haemoglobin preparation and either trypsin (1 μg) or α_2M (200 μg). Incubation for 1 h at 37°C was followed by addition of 0.5 ml of outdated newborn-calf serum (GIBCO, Grand Island, NY, U.S.A.) and 0.4 ml of 50% (w/v) trichloroacetic acid. After 30 min in ice the solution was cleared by centrifugation, and 1 ml of the supernatant was added to 15 ml of Instagel II (Packard). Radioactivity was counted in a Tri-Carb liquid-scintillation counter (Packard) with external standardization.

Active-site titration of bovine trypsin (1-chloro-4-phenyl-3-tosylamidobutan-2-one-treated; Worthington) was done with *p*-nitrophenyl *p*-guanidinobenzoate (ICN Corp., Cleveland, OH, U.S.A.) as described by Chase & Shaw (1970).

Results

Effect of trypsin on methylamine incorporation

In a first series of experiments we wanted to examine the quantitative relationship between trypsin binding to α_2M and the 'methylamine-reactive' sites. This was done by titration with [^{14}C]methylamine of the remaining sites in α_2M–trypsin preparations with different degrees of complex-formation.

By titration of α_2M with trypsin, a classical titration curve was constructed for trypsin-inhibitor-resistant trypsin activity (Fig. 1*a*). The plateau was reached at an α_2M/trypsin molar ratio of 1:3. In the linear part more than 95% of the trypsin added was not inhibited by soya-bean trypsin inhibitor. Samples of all data points, examined by polyacrylamide-gel electrophoresis, under denaturing and reducing conditions, showed progressive binding of trypsin to be accompanied by a decreasing amount of α_2M monomer (M_r 185000) and increased amounts of a protein band with apparent M_r 85000 (Fig. 1*b*). Bands at M_r 127000 and 61000 are due to thermal fragmentation of native α_2M (Harpel *et al.*, 1979). Bands at M_r 25000, 12000 and 10000 were also seen when free trypsin was run under similar conditions. A small amount of undissociated complexes remained in the upper part of the gel (Harpel *et al.*, 1979).

The different α_2M–trypsin preparations (corresponding to each data point in Fig. 1) were examined further in two ways: by addition of excess of ^{125}I-labelled trypsin (Van Leuven *et al.*, 1979) and by incubation with [^{14}C]methylamine. In both cases, α_2M was separated from excess of reagent by rate electrophoresis on 5% polyacrylamide gels, and the amount of radioactivity present in α_2M bands was determined. Untreated α_2M (no trypsin added) was used as a control. The results showed a close parallel between [^{14}C]methylamine incorporation and binding of ^{125}I-labelled trypsin over the entire titration curve. The sample at the plateau (α_2M fully saturated with trypsin) did not bind appreciable amounts of ^{125}I-labelled trypsin or of [^{14}C]methylamine. These results confirm and extend the finding that, after formation of complex with trypsin, the methylamine-reactive sites in α_2M are no longer reactive.

Active-site titration of trypsin showed the preparation to contain between 60 and 70% active enzyme, indicating the molar binding ratio of active trypsin to α_2M to be about 2:1. Incorporation of [^{14}C]methylamine was found to be between 3.7 and 4.1 mol/mol of α_2M (M_r 725000). Thus, for each molecule of trypsin bound, two methylamine-reactive sites were inactivated.

We have shown that [^{14}C]methylamine incorporation into α_2M displayed biphasic reaction kinetics with respect to time, and that incorporation was increased by formation of trypsin complex (Van Leuven *et al.*, 1981*a*,*b*). These two phenomena were examined in the same experiment: [^{14}C]methylamine incorporation was measured as a function of time;

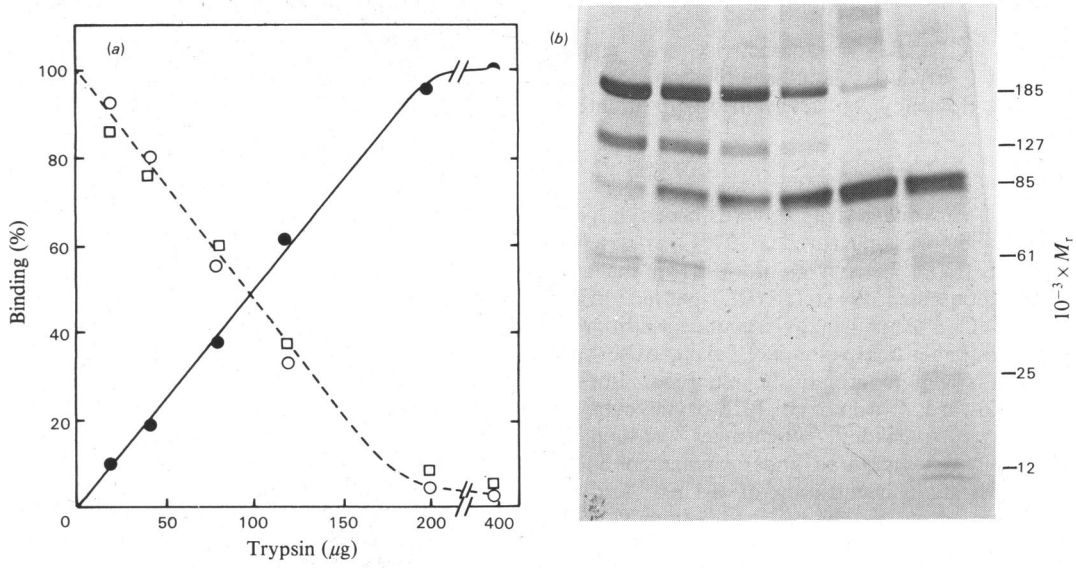


Fig. 1. *Titration of α_2M with trypsin, ^{125}I-labelled trypsin and [^{14}C]methylamine*

(*a*) Portions (2mg) of human α_2M were treated with the amounts of trypsin indicated in 25mM-Tris/HCl buffer, pH8.0. Samples were taken for the determination of trypsin-inhibitor-resistant amidase activity (●) and for polyacrylamide-gel electrophoresis (*b*). The equivalent of 750μg of α_2M from the original incubation mixtures was further treated with either excess of ^{125}I-labelled trypsin (for 15min at 25°C) or with 25mM-[^{14}C]methylamine (for 18h at 37°C). Radioactivity in α_2M was determined by excision and dissolution of stained bands after separation on 5% polyacrylamide gels. The data are expressed relative to completely saturated α_2M: for amidase activity (at 400μg of trypsin) activity measured was 0.026 A_{410} unit increase/min (for 100μg of α_2M). Native α_2M (no trypsin added) bound respectively 31240c.p.m. of ^{125}I-labelled trypsin and 6035c.p.m. of [^{14}C]methylamine per 10μg of protein. Binding is expressed relative to these results: ○, binding of ^{125}I-labelled trypsin; □, [^{14}C]methylamine incorporation. (*b*) Polyacrylamide-gel electrophoresis after denaturation and reduction. Samples (taken from titration mixtures, see above) were treated with sodium dodecyl sulphate and 2-mercaptoethanol (1% each, final concentrations) for 10min at 80°C. In each lane 20μg of α_2M for the data points in (*a*) was applied. A gradient gel (6–20% polyacrylamide) was used. Apparent M_r values ($\times 10^{-3}$) are indicated. A very similar pattern was obtained after [^{14}C]methylamine treatment of the samples, whereas after treatment with ^{125}I-labelled trypsin all samples ran as the M_r-85000 component.

to parallel incubation mixtures, also containing [^{14}C]methylamine, trypsin was added at the same time points. Incorporation of [^{14}C]methylamine again showed biphasic reaction kinetics (Fig. 2). Addition of trypsin was accompanied by an increased [^{14}C]methylamine incorporation into α_2M, but this was only significant in the first, rapid, phase of incorporation. The slow-phase component, previously shown to parallel the conformational change from electrophoretic slow-form into fast-form α_2M (Van Leuven *et al.*, 1981*a*), was not appreciably affected by formation of trypsin complex. When the preparations obtained after 2h incubation with [^{14}C]methylamine were kept under the same conditions for 18h, it was found that [^{14}C]methylamine incorporation had increased from 350 to 624c.p.m./μg of α_2M, as expected (Fig. 2). If the sample was treated with trypsin, incorporation of [^{14}C]methylamine remained at the 2h value (362c.p.m./μg). By sodium dodecyl sulphate/polyacrylamide-gel electrophoresis after denaturation and reduction, it was found that, in all samples treated with trypsin, α_2M was present as the M_r-85000 component (results not shown). These results indicate that, although formation of trypsin complex stimulates incorporation of [^{14}C]methylamine, once the complex is formed no further incorporation occurs.

Effect of methylamine on trypsin binding

In view of the foregoing and our previously described findings (Van Leuven *et al.*, 1981*a*,*b*), we wanted to examine further the inactivation of α_2M by methylamine as a function of time, with respect to trypsin binding and conformational change of α_2M. Change in conformation, indexed by the shift in electrophoretic mobility from slow-form to fast-form α_2M on 5% polyacrylamide gels, was studied with ^{125}I-labelled α_2M. Incubation with methylamine (25mM) decreased the amount of α_2M (^{125}I radioactivity) in slow form with time (Fig. 3). The radioactivity was quantitatively found in fast-form α_2M, inasmuch that at each data point over the entire

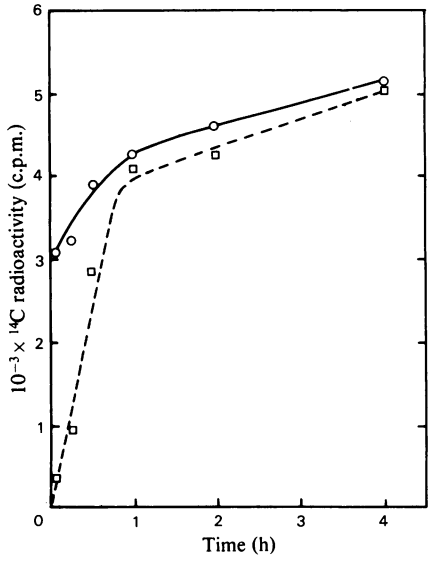


Fig. 2. *Time course of incorporation of [^{14}C]methylamine in α_2M and effect of formation of complex with trypsin*
Incubation of α_2M (2mg/ml) with 25mM-[^{14}C]-methylamine was in 50mM-Tris/HCl buffer, pH8.0, at 25°C. At the indicated time points, samples were either directly applied to 5% polyacrylamide gels or treated with trypsin (molar ratio to α_2M, 3:1), by addition of a concentrated solution of the enzyme (20mg/ml) so that dilution of methylamine was negligible. After 10min samples were applied to 5% polyacrylamide gels. After electrophoresis, radioactivity in α_2M was determined by excision of Coomassie-Blue-stained bands, dissolution in concentrated H_2O_2 and liquid-scintillation counting of radioactivity. □, Incorporation of [^{14}C]methylamine into native α_2M; ○, incorporation of [^{14}C]methylamine into α_2M by complex-formation with trypsin at the time points indicated. For the shortest incubation time (2min) results very similar to those depicted were obtained by adding α_2M last, to otherwise complete reaction mixtures, including trypsin or not (also see the text for details).

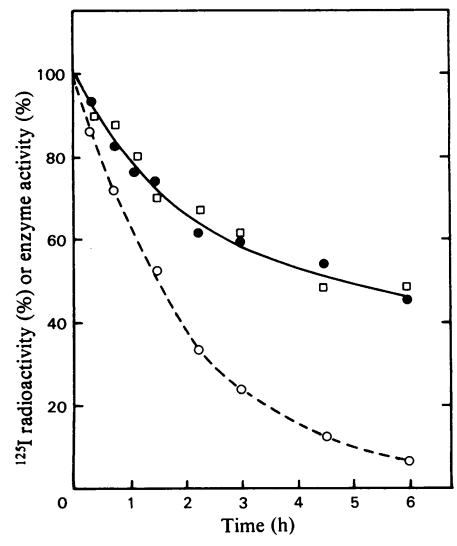


Fig. 3. *Time course of conformational change and trypsin binding of α_2M by methylamine*
^{125}I-labelled α_2M (1μg/ml) was incubated with 25mM-methylamine in 50mM-Tris/HCl buffer, pH8.0, at 25°C. At the indicated time points, samples were taken and analysed by rate electrophoresis in 5% polyacrylamide gels. Coomassie-Blue-stained bands of slow-form and fast-form α_2M were excised and radioactivity was determined (●, slow-form α_2M). In a parallel experiment native α_2M was incubated with methylamine under exactly the same conditions. At the indicated time points samples were taken and treated with excess of ^{125}I-labelled trypsin. Determination of radioactivity bound to α_2M was determined as described above and represents total binding of trypsin to α_2M (□). On the same samples enzymic activity was determined after addition of soya-bean trypsin inhibitor (○). Results are normalized to measurements with native α_2M, not treated with methylamine, incubated under otherwise identical conditions.

incubation period total radioactivity recovered from the gel was more than 92% of the amount applied. Kinetically, the time course of conformational change did not follow first-order kinetics with respect to time.

A similar experiment was done with unlabelled α_2M but under otherwise identical conditions, and at each time point excess of ^{125}I-labelled trypsin was added. By this treatment all samples were converted into fast-form α_2M. These preparations were then examined for the amount of trypsin bound, both by determination of radioactivity present in α_2M and by determination of trypsin activity not inhibited by soya-bean trypsin inhibitor. Radioactivity bound to α_2M decreased with the duration of incubation with methylamine, closely paralleling the conformational change (Fig. 3). However, trypsin-inhibitor-resistant trypsin activity decreased much more rapidly than was expected from the data on conformational change. After 6h of methylamine incorporation, trypsin-inhibitor-resistant binding was decreased to less than 10% relative to that with native α_2M incubated under identical conditions without methylamine. Kinetically, this time course was found to be first-order with respect to time. Taken together, these and previous results indicated methylamine incorporation, conformational change and trypsin binding to be different phenomena, related kinetically in a complicated fashion. Moreover, the difference between total binding of trypsin (^{125}I radioactivity) and trypsin-inhibitor-resistant binding showed that

Table 1. *Enzymic activity of trypsin and of α_2M–trypsin complexes*

Enzymic activity towards [^{3}H]acetylated haemoglobin and benzoylarginine *p*-nitroanilide was measured as detailed in the Experimental section. Complexes were prepared and isolated as described in the text. In the assay with haemoglobin activity is expressed as radioactivity (c.p.m.) not precipitated by trichloroacetic acid as liberated by 1 μg of trypsin or by the equivalent of 200 μg of α_2M as trypsin complexes. In the assay with benzoylarginine *p*-nitroanilide activity is expressed as increase in A_{410}/min with 10 μg of trypsin or the equivalent of 125 μg of α_2M as trypsin complexes. Benzamidine was used at a final concentration of 25 mM; 20 μg of soya-bean trypsin inhibitor (STI) was used per assay where indicated. Results are the means of duplicate determinations. Results from the assay with haemoglobin are corrected for a blank value of 1205 c.p.m. (no trypsin or α_2M added, but otherwise identical conditions).

	Assay with [^{3}H]acetylated haemoglobin (c.p.m.)	Assay with benzoylarginine *p*-nitroanilide (ΔA_{410}/min)
α_2M native	31	0.001
Trypsin	10409	0.040
+STI	108	0.001
+benzamidine	214	0.002
α_2M–trypsin	2636	0.029
+STI	1257	0.030
+benzamidine	673	0.002
α_2M–trypsin (methylamine)	8308	0.018
+STI	1243	0.009
+benzamidine	777	0.001

complexes thus formed are anomalous: some of the ^{125}I-labelled trypsin is inhibited by soya-bean trypsin inhibitor. Examination of the observation revealed that soya-bean trypsin inhibitor did not displace labelled trypsin from α_2M in these preparations, not even with prolonged incubation with the inhibitor (up to 6 h).

In the spectrophotometric assay for trypsin enzymic activity it was found that when these preparations were examined immediately after addition of soya-bean trypsin inhibitor, or when the trypsin inhibitor was added to the assay mixture in the cuvette, curvilinear tracings were obtained, which became linear only after about 30 min. This is in sharp contrast with either free trypsin or classical α_2M–trypsin complexes, for which linear time curves were always obtained, even when soya-bean trypsin inhibitor was added to the complete reaction mixture in the cuvette. With free enzyme, complete inhibition by soya-bean trypsin inhibitor was immediate on addition of the inhibitor under these conditions. Apparently the reaction with soya-bean trypsin inhibitor in these anomalous α_2M–trypsin complexes is slowed down considerably, indicating that trypsin was bound or entrapped by α_2M in a sterically hindered fashion, but still reactive towards soya-bean trypsin inhibitor.

These observations were taken into account, as the data on enzymic activity (Fig. 3) all pertain to the linear part of the reaction time course, whereas subsequent experiments (see below) were done after reaction with soya-bean trypsin inhibitor for at least 30 min.

Characterization of trypsin-inhibitor-inhibited complexes

The difference between total binding of trypsin (^{125}I radioactivity) and trypsin-inhibitor-resistant binding (enzymic activity) after methylamine treatment indicated formation of 'abnormal' complexes, in which trypsin could still be inhibited by soya-bean trypsin inhibitor. These complexes were further examined for proteolytic activity towards a high-molecular-weight proteinaceous substrate and for binding of soya-bean trypsin inhibitor to the active site of trypsin. This was done with [^{3}H]acetylated haemoglobin and ^{125}I-labelled soya-bean trypsin inhibitor. For these experiments α_2M was treated for 3 h with methylamine under conditions used for the kinetic studies described above. Control preparations were similarly treated, with omission of methylamine. At the end of the incubation, trypsin was added in excess to α_2M (molar ratio 4:1). The complexes were separated from excess of trypsin and methylamine by gel filtration on an Ultrogel AcA-34 (LKB) column (1 cm × 60 cm). Fractions containing α_2M were pooled and concentrated.

Proteolytic activity towards [^{3}H]acetylated haemoglobin was measured. A typical experiment is shown in Table 1, in which proteolytic activity towards [^{3}H]acetylated haemoglobin and amidase activity towards benzoylarginine *p*-nitroanilide are compared for different preparations and treatments.

Neither assay detected enzyme activity in native isolated α_2M. Control α_2M–trypsin complexes showed the typical behaviour when assayed with

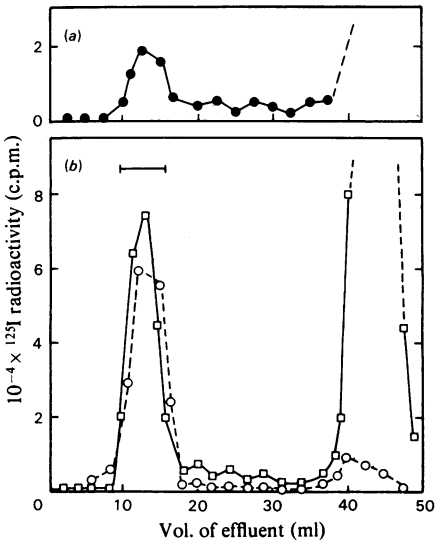


Fig. 4. *Binding of ^{125}I-labelled soya-bean trypsin inhibitor to α_2M–trypsin complexes*

α_2M–trypsin complexes were formed either with native α_2M or after treatment of α_2M with methylamine (see the text for experimental details). These complexes were further incubated for 60min with excess of ^{125}I-labelled soya-bean trypsin inhibitor and then analysed by gel filtration (Ultrogel AcA-34; column 1cm × 60cm; in 0.1M-Tris/HCl buffer, pH8.0); the flow rate was 6ml/h, and 1ml fractions were collected. In (*b*) distribution of radioactivity is shown as obtained with trypsin complexes of methylamine-treated α_2M (□). Fractions denoted by the horizontal bar contained α_2M as detected by 'rocket' immunoelectrophoresis. These fractions were pooled, concentrated and rechromatographed on a similar column (○). In (*a*) distribution of radioactivity is shown under similar conditions of the sample complexes but treated with 7-amino-1-chloro-3-tosylamidoheptan-2-one (2mM) before addition of ^{125}I-labelled soya-bean trypsin inhibitor. A pattern very similar to this was obtained with control α_2M–trypsin complexes (α_2M not treated with methylamine or with 7-amino-1-chloro-3-tosylamidoheptan-2-one). Equal amounts of α_2M (2mg) and radioactivity were loaded on the columns.

benzoylarginine *p*-nitroanilide: no inhibition of activity with soya-bean trypsin inhibitor and nearly complete inhibition with benzamidine, a low-molecular-weight inhibitor. With [^{3}H]acetylated haemoglobin low enzymic activity was detected, inhibited partially by soya-bean trypsin inhibitor and nearly completely by benzamidine. Inhibition comparable with that obtained with benzamidine was found with inhibitors Trasylol and 7-amino-1-chloro-3-tosylamidoheptan-2-one ('TLCK') (results not shown). The complexes generated and isolated after methylamine treatment [α_2M–trypsin (methylamine); Table 1] showed a much higher activity towards haemoglobin than did the control preparation, despite the fact that less trypsin was present as judged from the measurements of activity towards benzoylarginine *p*-nitroanilide. In the latter, soya-bean trypsin inhibitor inhibited 50% of the activity present, and benzamidine inhibited completely. Comparable activities were found for the control and the methylamine-treated complexes when assayed with [^{3}H]acetylated haemoglobin in the presence of soya-bean trypsin inhibitor or benzamidine (Table 1). These results indicate some proteolytic activity to be associated with α_2M–trypsin complexes, and that much more activity was found after methylamine treatment.

Binding of soya-bean trypsin inhibitor to α_2M–trypsin complexes was examined with ^{125}I-labelled trypsin inhibitor, added to complexes prepared after methylamine treatment as described above. Separation of α_2M complexes from excess of labelled soya-bean trypsin inhibitor was done by gel filtration (Fig. 4). A peak of radioactivity was present in the void volume of the column, closely paralleling the distribution of α_2M, as determined by 'rocket' immunoelectrophoresis, and clearly separated from the bulk of radioactivity. Fractions indicated by the horizontal bar were pooled, concentrated and re-run on a similar column. Although some redistribution of radioactivity was apparent (Fig. 4, broken line), more than 80% was recovered at a position identical with that of the first separation. Chromatography in similar conditions of methylamine–α_2M–trypsin complexes treated with 7-amino-1-chloro-3-tosylamidoheptan-2-one before additon of ^{125}I-labelled soya-bean trypsin inhibitor and of control α_2M–trypsin complexes both resulted in patterns characterized by a much decreased amount of radioactivity recovered in the α_2M peak (Fig. 4). By polyacrylamide-gel electrophoresis under native conditions of similar preparations, radioactivity was found associated with α_2M bands, both on 5% gels and on gradient gels (6–20%). However, radioactivity was also found in positions smeared between that of free ^{125}I-labelled soya-bean trypsin inhibitor and α_2M. This indicates that during electrophoresis dissociation of soya-bean trypsin inhibitor occurred.

As a final aspect of these anomalous complexes, we examined the extent of covalent binding of ^{125}I-labelled trypsin to α_2M under control conditions and after pretreatment with methylamine for 3h as described above. Covalent binding of the proteinase to α_2M, extensively reported elsewhere (Van Leuven *et al.*, 1981*b*), was determined as denaturation- and reduction-resistant labelled material, present as high-molecular-weight protein on polyacrylamide-gel electrophoresis. Radioautography indicated that

complexes between ^{125}I-labelled trypsin and α_2M pretreated with methylamine (25 mM) for 3 h did not contain appreciable amounts of covalently bound trypsin. Determination of radioactivity in excised bands of the gel showed that about 10% of the total ^{125}I-labelled trypsin bound to α_2M was in a covalent linkage as opposed to classical α_2M–trypsin complexes, in which 49% of the label was present as protein of apparent M_r higher than that of the free enzyme. Although in the procedure used in the present work methylamine remained present during formation of the complex, we have shown that at this concentration (25 mM) inhibition of covalent binding by the primary amine is far from complete (about 33%) (Van Leuven *et al.*, 1981*b*). This indicates that absence of covalent binding was typical for these anomalous complexes. Moreover, this correlates with our finding that no extra methylamine is incorporated by formation of complex in the slow phase of the methylamine reaction (Fig. 2). The absence of methylamine incorporation and of covalent binding to the 'methylamine-reactive' site under these conditions indicates that at least a third possible reaction at this site must be envisaged. Although simple hydrolysis is a likely candidate, we cannot exclude the possibility that internal reactions in the α_2M structure occur.

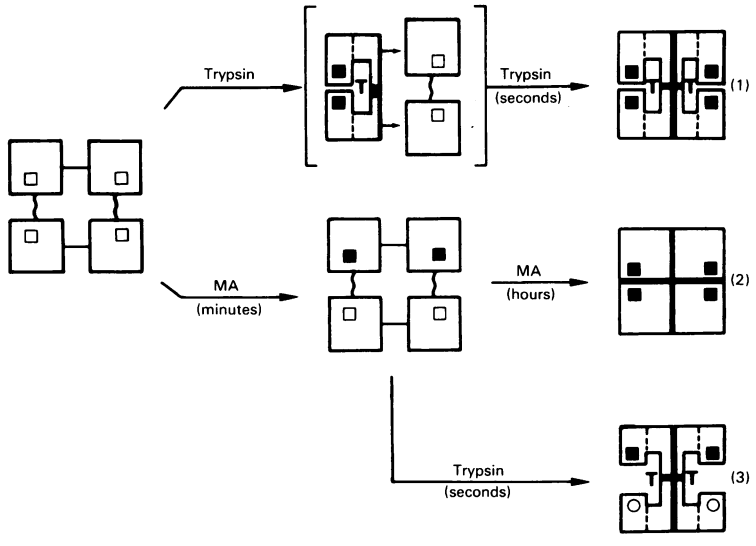
Discussion

The data on kinetics of interaction of trypsin and methylamine with human α_2M, presented in this paper, have to be considered in relation to the subunit structure of α_2M. From the available literature data (Harpel, 1977; Barrett *et al.*, 1979; Swenson & Howard, 1979; Harpel *et al.*, 1979) and from the present results, human α_2M can be represented as a tetramer of identical subunits (see Scheme 1). These are covalently linked in pairs by disulphide bridges, forming half-molecules, which in turn associate non-covalently to form the native tetramer. Each subunit contains one methylamine-reactive site, whereas two subunits must be involved to form one trypsin-binding site. In a trypsin complex one peptide bound per subunit is proteolytically cleaved and all methylamine-reactivity has disappeared. In this static picture of α_2M several important mechanistic questions remain, such as what two subunits form one trypsin-binding site; are there interactions between subunits during formation of complex and what is the sequence of events leading to a stable complex? Although not providing definite answers, the present results are considered in this respect.

Titration of α_2M with increasing amounts of trypsin and back-titration with [^{14}C]methylamine or ^{125}I-labelled trypsin showed a close parallel between the remaining methylamine-reactive and trypsin-binding sites in a 2:1 ratio. Moreover, these sites were kept intact to the expected proportion. Therefore these results indicate that, even at low trypsin/α_2M ratios, no unaccounted loss of trypsin-binding sites occurs. Binding of trypsin is accompanied by a conformational change of α_2M, resulting in an increased electrophoretic mobility. In these and other experiments we have never observed trypsin (^{125}I-labelled) in association with slow-form α_2M or binding of trypsin to α_2M already in the fast form. The reaction with trypsin thus is rapid and results practically immediately in electrophoretic fast-form α_2M. In conditions where the proteinase/α_2M ratio is low (as usually must be the case *in vivo*, and in titration experiments such as that reported in the present paper), this poses a problem for the second trypsin-binding site to react. Therefore we conclude from our data that reaction (and complex-formation) of trypsin at one site in α_2M renders the second site more susceptible (kinetically) to trypsin action than in native α_2M. Teleologically this situation would also seem the most efficient for a bivalent proteinase inhibitor *in vivo*. Probably neighbour interactions between subunits or half-molecules in the tetramer induce faster reaction at the second site once the first is occupied (Scheme 1), although we cannot exclude the possibility that a fast dissociation–reassociation of half-molecules occurs, preferentially re-forming tetramers composed of subunits in the same conformation (native or modified by trypsin).

The inactivation of α_2M with methylamine, although resulting in a final conformation identical with that of α_2M–trypsin complexes (Van Leuven *et al.*, 1981*a*), seems kinetically completely different. We have shown here and elsewhere (Van Leuven *et al.*, 1981*a*,*b*) that: (*a*) reaction with methylamine and trypsin are mutually exclusive when the treatment has already resulted in conformationally stable fast-form α_2M (however, see below); (*b*) during formation of trypsin complex the methylamine-reactive site is transiently activated; (*c*) incorporation of methylamine shows biphasic reaction rates, and labelled slow-form α_2M can be isolated.

Although we maintain our previously reached conclusion that the proteolytic event triggers activation of the methylamine-reactive site (Van Leuven *et al.*, 1981*b*), the present results show that bypassing the proteolytic event with methylamine actually seems to make the second half-molecule less reactive: slower incorporation of methylamine after about 50% of the sites have reacted and no appreciable incorporation of methylamine when trypsin is allowed to react in the slow phase of methylamine incorporation. Apparently, methylamine reacts rapidly with only two out of the four subunits in the tetramer. As this results in a completely different reactivity of the other subunits (in



Scheme 1. *Schematic representation of the different reactions of trypsin and methylamine with α_2M*

This scheme is an attempt to visualize our findings in this and previous papers (Van Leuven *et al.*, 1981*a*,*b*). The representation of α_2M as a tetramer is based on literature data (Barrett *et al.*, 1979; Swenson & Howard, 1979; Harpel *et al.*, 1979; and references cited therein). Monomers of apparent M_r 185000 are represented by the large squares. These are held together by disulphide bridges to form half-molecules, which combine in turn non-covalently to form the tetramer. As it is unknown what two subunits make up one trypsin-binding site, the connections drawn between monomers can either be covalent (disulphide) or non-covalent. For the model, this does not make any difference. The 'methylamine-reactive' sites are represented by small open squares. Reaction (1) represents formation of complex with trypsin (T): the two available sites are filled up in rapid succession. The intermediate form (between square brackets) is not observed. Inactivation of methylamine-reactive sites is denoted by filled squares. Arrows between monomers in this form represents the postulated interactions between subunits (see the text). Broken lines denote proteolytic cleavage of the monomers. Reaction (2) represents the inactivation of α_2M by methylamine (MA). The intermediate form is still slow-form α_2M, which has reacted with methylamine and can be isolated. Probably this form, when treated with trypsin (3), leads to formation of anomalous complexes. These have proteolytic activity towards haemoglobin, and trypsin can bind soya-bean trypsin inhibitor to its active site. The open circles denote the third possible reaction at the methylamine-reactive site: although these sites were inactivated, they do not bind trypsin or methylamine. Some of the sites in a trypsin complex (1) also must react in this fashion, as covalent binding is never complete, even in the absence of methylamine. Approximate reaction rates are given (time to complete reaction). The final forms of α_2M, obtained by the different treatments, are extensively characterized and discussed in the text. The thick lines between monomers in these complexes represent the increased non-covalent interactions, as pointed out by Barrett *et al.* (1979).

contrast with trypsin), our data indicate that methylamine inactivates two subunits (in different half-molecules) that do not make up a trypsin-binding site (Scheme 1). The symmetry in α_2M and the excess of methylamine used (as opposed to titration with sub-saturating amounts of trypsin) are in favour of such a mechanism.

Further interpretation of the present results is difficult without becoming highly speculative about the nature of the mechanism and the sequence of events leading to an α_2M–proteinase complex. Our results do indicate, however, that this sequence must be strictly adhered to, because reversing the normal sequence (proteolytic event followed by methylamine-reactive-site reaction) leads to 'abnormal' complexes. These complexes exhibit proteolytic activity, and bind soya-bean trypsin inhibitor tightly (although slowly) to the active site of trypsin (as shown by inhibition by 7-amino-1-chloro-3-tosylamidoheptan-2-one). These are sufficient criteria to establish the presence of 'trypsin-inhibitor-inhibited' α_2M–trypsin complexes. Further experiments to characterize their nature have so far been unsuccessful; these complexes did not bind to immobilized soya-bean trypsin inhibitor, probably owing to steric factors. In isoelectric focusing, their pattern was different from that of classical α_2M–trypsin complexes, but overlays with agarose containing casein did not enable us to assign proteolytic activity to specific bands, owing to loss of resolution in the

agarose gels. Experiments on receptor-mediated endocytosis by fibroblasts in culture are hampered by lack of specific labelling of the 'trypsin-inhibitor-inhibited' complexes.

Further experimental work is needed to examine these aspects and the mechanism of formation of α_2M complexes. Eventually these will allow us to determine the biological significance, if any, of the present observations, under normal or pathological conditions *in vivo*.

This will certainly be facilitated by our finding that a neo-antigenic site on α_2M complexes is closely related to the receptor-recognition site on these complexes. Evidence for this was obtained by use of monoclonal antibodies (Marynen *et al.*, 1981).

While this work was in progress, several reports appeared tentatively identifying the 'methylamine-reactive site' in α_2M as a thioester, formed between a γ-glutamyl residue and a cysteine group (Tack *et al.*, 1980; Sottrup-Jensen *et al.*, 1980; Howard, 1981). Although this does not allow us to interpret our findings further, it might offer alternative experimental approaches to examine the mechanism and the significance of the findings described in the present paper.

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