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NON-PRODUCTIVE ACTIVATION OF THE PROTEINASE BINDING SITES OF α_2-MACROGLOBULIN ON REACTION OF THE INHIBITOR WITH MATRIX-LINKED TRYPSIN

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SUMMARY: The reaction of α_2-macroglobulin with matrix-linked trypsin is accompanied by apparently identical "bait" region cleavage, thioester cleavage and conformational change as the reaction with the soluble enzyme. However, no binding of proteinase occurs, but instead the inhibitor loses its ability to inactivate soluble trypsin. These findings indicate that the proteinase binding sites of α_2-macroglobulin are activated in the normal manner but decay before being able to bind the immobilized enzyme. Such non-productive activation of binding sites may occur also in the reactions of α_2-macroglobulin with soluble proteinases, thus explaining apparent enzyme inhibitor stoichiometries of less than 2:1 observed in these reactions.

α_2-Macrogobulin is a high-M_r, tetrameric plasma protein that acts as an inhibitor of a wide variety of proteinases (1,2). The inactivation appears to be triggered by restricted proteolysis of a "bait" region of α_2M, leading to cleavage of a thioester bond in each subunit and to a conformational change that creates a binding site for the proteinase (1,3-10). The enzyme binds to this site in a manner that abolishes its activity only against macromolecular substrates (1,11). The inhibitor may thus physically entrap the proteinase (1).

Most evidence indicates that each α_2M tetramer has the capacity to bind two molecules of small proteinases (4-6,11-15). However, apparent stoichiometries of less than 2:1 have frequently been observed also with such enzymes (4-6,14,15). Moreover, larger proteinases bind to α_2M with a stoichiometry close to 1:1 (12-14). This paper shows that reaction of α_2M with matrix-linked trypsin activates the proteinase binding sites of the inhibitor in an apparently normal manner without leading to binding of the enzyme. Similar non-productive activation of the binding sites may account for apparent stoichiometries of less than 2:1 observed also with enzymes in solution.

Abbreviations: α_2M, α_2-macroglobulin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

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MATERIALS AND METHODS

Human α_2M was purified and its concentration was determined as described previously (10). Bovine trypsin (EC 3.4.21.4; type III, Sigma Chemical Co., St. Louis, MO) was coupled to CNBr-activated Sepharose 4B (Pharmacia Ltd., Uppsala, Sweden) at pH 8.5; the resulting protein content was 9.1 mg per ml wet gel. The amount of active trypsin bound to the gel, determined by spectrofluorimetric active-site titration with 4-methylumbelliferyl p-guanidinobenzoate in 0.1 M phosphate, pH 6.0 (16,17) was 185 nmoles per ml wet gel, indicating that about 45% of the bound enzyme was active.

Proteolytic cleavage of α_2M was monitored by dodecyl sulfate/polyacrylamide gel electrophoresis under reducing conditions (5,15,18). The extent of cleavage was expressed as the intensity (measured by scanning the gels after staining with Coomassie Brilliant Blue R-250) of the M_r ~85000 band as a percentage of the intensities of all bands on the gel. The conformational change of α_2M on reaction with enzymes (9,10) was analysed by polyacrylamide gradient gel electrophoresis (4). Commercial 4-30% (w/v) gels (Pharmacia) were used after preelectrophoresis to remove the ammonium sulfate present in the gels.

The trypsin-binding capacity of α_2M was measured by saturation of α_2M with trypsin and inactivation of excess free enzyme by soybean trypsin inhibitor (1,11,15). The activity of the α_2M-bound trypsin was then measured with the chromogenic substrate N-benzoyl-L-isoleucyl-L-glutamylglycyl-L-arginine p-nitroanilinide (S-2222; KabiVitrum, Stockholm, Sweden). Actual α_2M-associated trypsin activity in samples reacted with trypsin-Sepharose was measured in the same way but without the addition of trypsin. Total trypsin activity in these samples was analysed by also omitting the soybean trypsin inhibitor.

Sulfhydryl groups were determined by reacting α_2M (at a concentration of 0.4-0.5 mg/ml, i.e. 0.5-0.7 μM) with 0.5 mM [1-^{14}C]iodoacetic acid (specific activity 0.41 MBq/μmol) in 0.05 M Hepes, 0.1 M NaCl, 2 mM EDTA, pH 7.4, for at least 60 min. The number of moles of sulfhydryl groups reacted/mole of α_2M were measured after extensive dialysis against the buffer.

Sulfhydryl groups were also analysed with the fluorogenic reagent N-(7-dimethylamino-4-methyl-3-coumarinyl)maleinimide (15,19) in the same Hepes buffer. The final concentrations of α_2M and reagent were 0.1 mg/ml (~0.14 μM) and 10 μM, respectively. The number of moles of sulfhydryl groups exposed/mole of α_2M was estimated by comparing the fluorescence of the sample with that produced by an α_2M standard in the presence of an excess of trypsin. Four sulfhydryl groups were assumed to be exposed/mole of α_2M in the standard (6,15).

RESULTS

α_2M (final concentration 0.5 mg/ml, i.e. ~0.7 μM) was mixed with trypsin-Sepharose to a concentration of active trypsin in the suspension of 25 μM. The buffer was 0.05 M Hepes, 0.1 M NaCl, 2 mM EDTA, pH 7.4. Aliquots of the suspension were incubated for increasing times at 37°C with gentle stirring and were then centrifuged to remove the trypsin-Sepharose. At least ~75% of the protein was recovered in the supernatant in all samples (Fig 1). The α_2M in the supernatant was slowly cleaved by the matrix-linked trypsin to fragments electrophoretically identical with the M_r ~85000 fragments produced in the reaction of α_2M with proteinases in solution (Figs 1 and 2) (3-5). The ability of the α_2M in the supernatant to bind trypsin decreased concurrent with the proteolytic cleavage. However, only minimal amounts of trypsin were found to be associated with the α_2M in the supernatant. Analyses of total trypsin activity showed no detectable amounts of free enzyme.

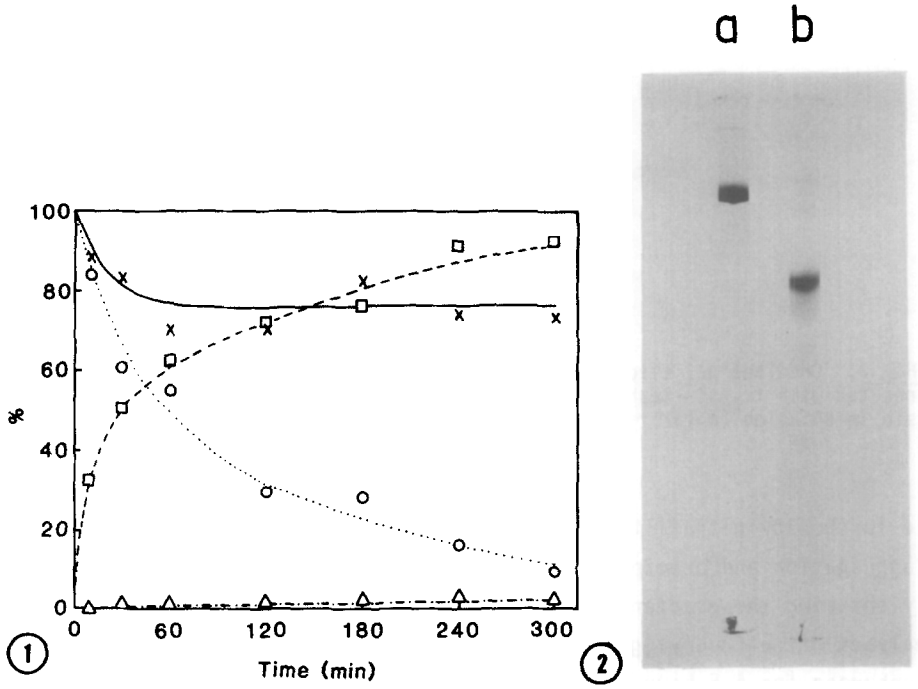

Fig 1. Reaction of α_2M with trypsin-Sepharose. (x—x), α_2M recovered in supernatant, in per cent of amount added; (□—□), α_2M in supernatant cleaved to M_r ~85000 fragments, in per cent of total protein; (o····o), trypsin-binding capacity of α_2M in supernatant, in per cent of the trypsin-binding capacity of the same amount of intact α_2M; (△-·-·△), trypsin activity associated with α_2M in supernatant, in per cent of the maximal trypsin-binding capacity of the same amount of intact α_2M.

Fig 2. Dodecyl sulfate/polyacrylamide gel electrophoresis of: (a) intact α_2M; (b) α_2M reacted with trypsin-Sepharose for 4 h. 7.5 µg protein was applied per gel.

A decreased amount of trypsin-Sepharose resulted in a proportionally slower rate of proteolysis. A high ratio of matrix-linked trypsin to α_2M was thus required for the reaction to occur at a reasonable rate, indicating that the reactivity of the immobilized enzyme with the α_2M "bait" region was low. Control experiments with ethanolamine-Sepharose gave no cleavage or decrease in activity of α_2M. The recovery of α_2M in these experiments was ~90%. The lower recovery with trypsin-Sepharose may be due to a small amount of binding of the α_2M to the matrix-linked trypsin or to precipitation of the cleaved α_2M (see below). Incubation of trypsin-Sepharose alone at 37°C showed a slow release of trypsin activity into the supernatant, comparable to the increase in α_2M-associated trypsin activity in Fig 1.

α_2M was incubated for 5 h with trypsin-Sepharose as in Fig 1, and the supernatant was analysed by gradient gel electrophoresis (Fig 3). Most of the protein migrated identically with α_2M that had reacted with trypsin in solution, i.e. as the fast electrophoretic band (4). Attempts to concentrate the

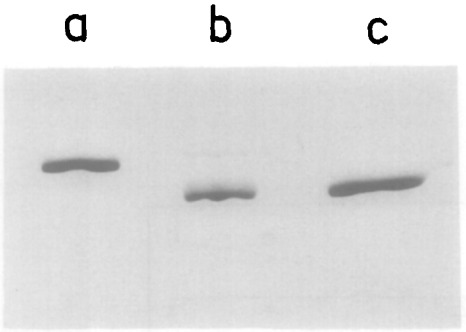

Fig 3. Gradient gel electrophoresis of: (a) intact α_2M (15 µg); (b) α_2M reacted with trypsin-Sepharose for 5 h (10 µg); (c) α_2M reacted with trypsin in solution (molar ratio trypsin:α_2M = 4:1) for 5 min (15 µg).

sample further by ultrafiltration before electrophoresis resulted in extensive aggregation and precipitation of the protein, as evidenced by material barely entering the gradient gel and by loss of protein.

Analyses of sulfhydryl groups in α_2M that had been incubated with trypsin-Sepharose for 4-5 hours showed 1-1.5 groups/mole of protein by both the radiolabelling and the spectrofluorimetric procedure. Addition of an excess of trypsin in solution to the samples after the reaction with the matrix-linked enzyme gave no significant increase in the number of reactive sulfhydryl groups. Incubation of α_2M with the immobilized trypsin in the presence of [^{14}C]iodoacetic acid increased the number of sulfhydryl groups that reacted with this reagent to about 2 moles/mole of α_2M. These findings indicate that all sulfhydryl groups that appear in α_2M on reaction with trypsin in solution (about 4 moles/mole of α_2M) (6) were released also in the reaction with the matrix-linked enzyme, but that extensive oxidation occurred during the long incubation. Such oxidation has been reported previously for sulfhydryl groups released in the reaction of α_2M with soluble trypsin (20).

DISCUSSION

Together, the results suggest that all events accompanying the reaction between α_2M and proteinases in solution, i.e. "bait" region cleavage, thioester cleavage and conformational change (1-10), occur also in the reaction with matrix-linked trypsin. However, the inhibitor does not bind the immobilized enzyme, but instead loses its ability to inactivate soluble trypsin. These observations indicate that the proteinase binding sites of α_2M are activated in the normal manner by the matrix-linked trypsin but are unable to bind the immobilized enzyme and therefore decay. Thus, the α_2M "trap" (1) may close before the proteinase is caught. The inability of α_2M to bind the

matrix-linked trypsin may be due to steric hindrance, to the severely restricted diffusion rate of the immobilized enzyme or to the slow cleavage rate. Similar activation of the proteinase binding sites of α_2M without subsequent binding of the proteinase may occur also with soluble enzymes, thereby explaining the stoichiometries of less than 2:1 that have been observed with proteinases in solution (4-6,12-15). Such non-productive activation of the binding sites may be more pronounced for larger enzymes, e.g. thrombin and plasmin, that cleave α_2M slowly and bind with approximately equimolar stoichiometry (12-14). A somewhat related proposal has been made recently on the basis of other evidence to explain proteinase-α_2M stoichiometries of less than 2:1 (14).

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