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HUMAN α_2-MACROGLOBULIN AS AN INHIBITOR OF INSOLUBLE TRYPSIN

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α_2-Macroglobulin binds to insoluble trypsin bound on agarose beads inducing a reduction of proteolytic activity of the enzyme towards large substrates such as azocasein. When trypsin was bound on other matrices like sheep red blood cells or latex beads, the inhibition of proteolytic activity by α_2-macroglobulin was complete. These results show that α_2-macroglobulin inhibits similarly both soluble and insoluble proteinases.

The Mr 720,000 plasma glycoprotein α_2-macroglobulin (α_2M) has been shown to inhibit a wide variety of proteinases (1). The inhibition is triggered by proteolysis of a specific bound within the α_2M subunit, resulting in the formation of enzyme-inhibitor complexes (2-5). It has been postulated that the inhibitor physically entraps the proteinase (1). The proteinase activity is not abolished following this process as hydrolysis of small substrates still occurs, although cleavage of large proteins is inhibited by putative steric hindrance (6). α_2M has been claimed to scavenge the blood circulation from active proteinases, especially by macrophage-mediated endocytosis (7-9).

α_2M has almost been exclusively studied using soluble proteinases. To our knowledge, only one study has approached the mechanism of reaction of α_2M with matrix-linked proteinases (10). In the latter paper it was concluded that the reaction of α_2M with insoluble trypsin was similar to that with soluble enzyme, i.e. the α_2M subunit was cleaved and conformational change occurred, although the binding of α_2M to insoluble trypsin was not observed.

Since membrane-bound proteinases with active site exposed to the extracellular environment, that could be in contact with α_2M have now beeen found in various cells, tissues and organisms (11-14), we have re-investigated whether α_2M could both bind and inhibit insoluble proteinase. In order to assess a possible interaction between α_2M

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and a proteinase, we choose a simplistic model in which trypsin was associated both covalently and electrostatically with various matrices including agarose beads, latex beads and sheep red blood cells.

MATERIAL AND METHODS

Trypsin (Worthington, New Jersey, USA) was active-site titrated (15). α_2M was prepared from fresh human plasma (16). α_2M was found to protect 1.7 to 2.0 mol trypsin/mol α_2M from inhibition by soy bean trypsin inhibitor (SBTI), using S2302 (Kabi Vitrum, Stockholm, Sweden) as substrate (17). α_2M- trypsin complex (α_2M-T) was prepared by mixing trypsin and α_2M (3mol/mol) in 25 mM sodium phosphate buffer pH 7.3 containing 150 mM NaCl. The unreacted enzyme was removed by chromatography through Zn-chelate sepharose (Pharmacia, Uppsala, Sweden). After reaction with trypsin, the transition from slow- to fast-configuration of α_2M was complete (18).

α_2M was radiolabelled (0.48 atom^{125}I/mol α_2M) using the Bolton and Hunter reagent (Radiochemical Center, Amersham, England) (19). [^{125}I]α_2M was fully active, since it changed completely to fast-configuration after reaction with trypsin. [^{125}I]α_2M-T complex was prepared as described above for native α_2M-T.

Insoluble trypsin attached to beaded agarose was purchased from Sigma, St-Louis, Missouri. Trypsin was coupled to sheep red blood cells (SRBC) before each experiment using $CrCl_3$ as coupling agent (20). Trypsin-coated latex beads were prepared freshly for each experiment by passive adsorption of trypsin (100 µl at 10 mg/ml) on latex beads (100 µl) (Sigma) in 0.1 M sodium barbital buffer pH 8.3 containing 20 mM $CaCl_2$ (buffer A). After 20 min incubation the excess of trypsin was washed off with the same buffer. Trypsin bound on SRBC and latex beads was not quantitated.

For binding studies, 10 µl of packed trypsin-agarose beads or control agarose beads were incubated for 30 min at room temperature with 100 nM [^{125}I]α_2M or 100 nM [^{125}I] α_2M-T in 50 µl Hank's balanced salt solution containing 2 mg/ml bovine serum albumin (BSA). The incubation mixtures were centrifuged at 13,000 g and the pellets extensively washed and counted in an automatic gamma counter.

For inhibition of insoluble trypsin by α_2M using azocasein as substrate, 25 µl aliquots of trypsin-coated SRBC or latex beads, were incubated with 25 µl of either α_2M, α_2M-T or BSA at a concentration of 2mg/ml for 30 min at room temperature. The reactants were washed off and 450 µl of 4 mg/ml azocasein in buffer A were added for another 30 min at 37°C. The reaction was stopped with 500 µl 5% trichloracetic acid, centrifuged at 13,000 g and the optical density of the supernatant was measured at 366 nm (21).

To show that α_2M protects insoluble trypsin from inhibition by SBTI, 25 µl aliquots of trypsin-coated latex beads or SRBC were incubated with 25 µl of 2mg/ml of α_2M or BSA for 30 min at room temperature. Then 10 µl of either 1 mg/ml SBTI or buffer A were added for 5 min. To 10 µl of these reaction mixtures, 300 µl of buffer A together with 50 µl of 4mM S2302 substrate were added. After 30 min incubation at 37°C, the reaction was stopped by addition of 500 µl of 5% trichloroacetic acid. After centrifugation at 13,000 g, the optical density of the supernatants was measured at 405 nm.

RESULTS

Binding of [^{125}I]α_2M to trypsin attached to cross-linked agarose.

As shown in Table 1, [^{125}I]α_2M bound specifically to trypsin immobilized on agarose beads. This binding required native unreacted [^{125}I]α_2M since the preformed [^{125}I]α_2M-T complex failed to bind to insoluble trypsin. The [^{125}I]α_2M binding to immobilized trypsin was specific as shown by competition experiments with a 50-fold molar excess of

Table 1 : Binding of [^{125}I]α_2M to insoluble trypsin

Additions	cpm bound to agarose beads coated with trypsin	uncoated
[^{125}I]α_2M [a]	184,000	49,000
[^{125}I]α_2M-T [a]	24,000	31,000
[^{125}I]α_2M + α_2M [ab]	43,000	31,000

a 890,000 cpm added in the reaction mixture.
b competition experiment carried out in the presence of 5 µM unlabelled α_2M.

unlabelled α_2M that reduced [^{125}I]α_2M binding to a level similar to non specific binding observed with agarose beads devoid of trypsin.

Although trypsin was in molar excess towards [^{125}I]α_2M, the inhibitor was not completely bound to trypsin-agarose beads (148,000 cpm bound versus 890,000 cpm added in the reaction). The unbound [^{125}I]α_2M appeared under both slow and fast configurations in rate electrophoresis(18)(data not shown).

Inhibition of proteolytic activity generated by insoluble trypsin in the presence of α_2M.

To test whether insoluble trypsin was inhibited after complex formation with α_2M, the generation of acid-soluble azocasein peptides catalyzed by insoluble trypsin was measured in the presence of α_2M, α_2M-T and BSA. We used an assay previously described for soluble enzyme (21), in which, after digestion of azocasein, acid-insoluble material is discarded and the supernatant measured for its peptidic content.

Since trypsin was not quantitated on both SRBC and latex beads, in the following experiments α_2M was first titrated against a fixed amount of reactants. A concentration

Table 2 : **Inhibition of insoluble trypsin by α_2M using azocasein as substrate**

Additions	**Optical density** (366 nm) Latex beads Exp. 1	Latex beads Exp. 2	SRBC Exp. 1	SRBC Exp.2	Agarose beads
Buffer	0.601	0.760	0.189	0.360	N.D.
BSA	0.585	0.762	0.165	0.380	0.620
α_2M-T	0.604	0.726	0.157	0.372	N.D.
α_2M	0.037	0.126	0.015	0.060	0.270

N.D., not determined.

Table 3 : **Protection by α_2M of insoluble trypsin from inhibition by SBTI**

	Optical density (405 nm)			
	Latex beads		SRBC	
Additions	Exp. 1	Exp. 2	Exp. 1	Exp.2
Buffer	1.285	1.152	0.651	0.645
BSA	1.264	1.098	0.701	0.643
α_2M	1.247	1.145	0.694	0.637
Buffer+SBTI	0.101	0.097	0.166	0.143
BSA+SBTI	0.065	0.102	0.165	0.145
α_2M+SBTI	1.191	1.095	0.593	0.609

of 2mg/ml α_2M was found to be in excess with respect to trypsin. Table 2 shows two experiments in which the proteolytic activity of trypsin-coated SRBC or latex beads was measured. Proteolytic activity was virtually completely inhibited in presence of α_2M. In contrast, preformed α_2M-T complex was unable to inhibit tryptic activity towards azocasein substrate, demonstrating that the inhibitory site of α_2M was required for the interaction with insoluble trypsin.

Similarly we used trypsin-agarose beads. The amount of active trypsin bound to the beads was titrated against a standard curve measuring soluble trypsin activity towards azocasein. An aliquot of trypsin-agarose beads corresponding to 1 µg soluble trypsin was incubated with 30 µg α_2M (2 molar excess) or BSA. In these conditions, the optical density measured at 366 nm decreased from 0.620 without α_2M to 0.270 with α_2M. Even with α_2M concentrations exceeding largely 2 molar with respect to trypsin, no additional inhibition was found (Table 1).

Protection afforded by α_2M in the esterase activity of insoluble trypsin in the presence of SBTI.

It is shown in Table 3 that in the absence of SBTI, trypsin-coated latex beads or SRBC cleaved S2302 independently of the presence of α_2M in the reaction mixture. When SBTI was added to the controls (buffer or BSA) a marked decrease of trypsin activity was observed, indicating that insoluble trypsin was inhibited by SBTI. In contrast, SBTI did not affect the esterase activity of trypsin after incubation with α_2M on both trypsin-coated SRBC or latex beads.

DISCUSSION

The results presented in this paper show that α_2M is able to bind to insoluble trypsin and to inhibit its proteolytic activity, partially when trypsin was coupled to agarose

beads and totally when trypsin was presented on SRBC or latex beads. These results do not seem to be in accordance with those presented by Björk. The latter author, using trypsin immobilized on Sepharose, concluded that the binding of α_2M did not occur, although the inhibitor was cleaved and inactivated in the supernatant (10). However in the reported experiment 10-20% of binding of α_2M to the beads was present (Fig.1 in ref. 10). Similar findings were found in our study when about 20% of radioactive α_2M was bound when trypsin agarose beads were used.

After extensive washing of the trypsin-agarose beads reacted with α_2M, this preparation remained effective in decreasing the proteinase activity of trypsin towards azocasein, showing thus that α_2M could react with a proteinase covalently linked to a matrix and inhibit its enzymatic activity. However bound α_2M was not totally effective in inhibiting trypsin activity of agarose-treated beads. In contrast, when the enzyme was coupled to SRBC or latex beads, the inhibition of insoluble trypsin by α_2M was virtually complete. These data thus show that the reactivity of α_2M with insoluble protease is dependent on the solid support to which trypsin is coupled. This could be due to the conformation of the insoluble enzyme or to steric hindrance by the matrix.

α_2M seems to inhibit insoluble trypsin in a manner similar as soluble trypsin or other proteinases (1,6,22-25). Free or insoluble α_2M-T complexes both retain the enzymatic activity towards low molecular weight substrates although hydrolysis of larger proteins is inhibited. Furthermore, protection of the enzymatic activity by α_2M against inhibition by SBTI is effective with both forms of enzyme. Both functional assays used in this study strongly argue for identical pattern of reaction, whatever the physical state of trypsin may be.

In order to establish a possible interaction of α_2M with an insoluble proteinase, we used a simplistic model in which trypsin was both covalently and electrostatically linked to a solid matrix. Further studies will indicate whether this particular interaction could also be extended to cellular membrane-bound proteinases. Although most of the membrane-bound proteinases that have been described so far are located inside the cell, thus inaccessible to plasmatic α_2M, membrane-bound proteinases, with active site exposed to the extracellular environment, have now been found in many cellular types. In particular, CALLA antigen is an active enzyme present at the surface of some leukemic and melanoma cell lines (11). CALLA antigen has been shown to be identical to human neutral endopeptidase NEP, a membrane-bound metalloproteinase with active site directed towards the extracellular environment, and present in several normal tissues (12). Ectoenzymes linked to the outer membrne have also been described in invading microorganisms such as Escherichia coli (13) or Leishmania (14). Furthermore, it is suggested that membrane-bound proteinases could be implicated in the killing mechanisms by carcinoma cell lines (26), neutrophils (27) or T lymphocytes (28,29).

Since α_2M is able to form complexes with a wide variety of proteinases (1), any contact of this inhibitor with proteinases embedded in complex membranes could perhaps modulate their proteolytic activity.

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