Relation of internal thioesters to conformational change and receptor-recognition site in α_2 -macroglobulin complexes

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The unique steric type of inhibition of endopeptidases by human α_2 -macroglobulin (α_2 M) and the inactivation of the latter by methylamine were examined in relation to the internal thioesters in α_2 M. The present results confirm our previous findings that disruption of the internal thioesters, is not in itself sufficient to cause the conformational change of α_2 M typical of α_2 M–proteinase complexes; the electrophoretically slow form of α_2 M with [14 C]methylamine incorporated was isolated. Moreover, this group is stabilized by derivatization of the exposed cysteine thiol groups. Cyanylation with 2,4-dinitrophenyl thiocyanate during the methylamine reaction was the most effective procedure, yielding essentially only slow-form α_2 M. Other thiol-specific reagents were less effective. A different reactivity was observed for half of the cysteine residues in α_2 M complexes. When allowed to react with trypsin the cyanylated derivative (slow-form α_2 M with thioesters broken) produced anomalous complexes; only half the expected amount of trypsin was bound, whereas the complexes were fully inhibited by soya-bean trypsin inhibitor and were proteolytically active. Despite this, the anomalous complexes were recognized by two highly specific probes: the fibroblast α_2 M-complex receptor and the monoclonal antibody (F2B2) directed against the receptor-recognition site on α_2 M complexes. The results show that the internal thioesters in α_2 M are necessary for the conformational change producing sterically inhibited endoprotease complexes, but do not participate as such in receptor-mediated endocytosis of these complexes.

α_2 -Macroglobulin is a unique proteinase inhibitor in the circulation of mammals because of its steric mode of inhibition of virtually all endoproteases, preserving the catalytic site of the enzymes in the complexes (Starkey & Barrett, 1977). Resulting α_2 M–proteinase complexes are rapidly cleared from blood and interstitial fluids by receptor-mediated endocytosis by fibroblasts (Van Leuven *et al.*, 1979), macrophages (Debanne *et al.*, 1975; Kaplan & Nielsen, 1979) and probably Küpffer cells (Ohlsson & Skude, 1976). The receptor is highly discriminative and recognizes specifically α_2 M–proteinase complexes (Van Leuven *et al.*, 1979; Kaplan &

Nielsen, 1979). We have previously shown that α_2 M, after inactivation by primary amines (methylamine; Steinbuch *et al.*, 1968), is recognized and internalized by fibroblasts in culture by the same mechanism as α_2 M–trypsin complexes (Van Leuven *et al.*, 1981a). Furthermore, the methylamine derivative proved, by criteria of rate electrophoresis and isoelectric focusing, to be in a conformation indistinguishable from proteinase complexes (Van Leuven *et al.*, 1981a).

To examine this structure of complexed α_2 M recognized by the cellular receptor, we have prepared monoclonal antibodies towards methylamine-inactivated α_2 M. One hybridoma was found to produce an antibody specifically reactive with complexed α_2 M (either proteinase- or methylamine-generated) and thereby inhibiting receptor-mediated endocytosis of the complex. Extensive characterization and further evidence that this antibody binds at the receptor-recognition site on α_2 M–complexes was obtained (Marynen *et al.*, 1981). Evidence has been presented that methylamine, as well as other primary amines, reacts covalently with α_2 M at a

Abbreviations used: α_2 M, α_2 -macroglobulin; Nph₂SCN, 2,4-dinitrophenyl thiocyanate; Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid); iPr₂P-F, di-isopropyl fluorophosphate; Tos-Phe-CH₂Cl, tosylphenylalanyl-chloromethane ('TPCK'); Bz-Arg-pNA, benzoylarginine p-nitroanilide; α_2 M.MA.CN, α_2 M methylamine-inactivated in the presence of Nph₂SCN; α_2 M.MA, methylamine-inactivated α_2 M; Tos-Lys-CH₂Cl, tosyl-lysyl-chloromethane ('TLCK').

reactive internal thioester (Swenson & Howard, 1979; Tack *et al.*, 1980; Sottrup-Jensen *et al.*, 1980).

In the present work we examined the reaction of methylamine with $\alpha_2\text{M}$, resulting in the exposure of the cysteine thiol groups, in relation to the conformational change and the exposure or assembly of the receptor recognition site, both typical for proteinase complexes. The results indicate that the cysteine thiol group, liberated by methylamine reaction, participates in the conformational change of $\alpha_2\text{M}$, but not in the recognition of the complex by the cellular receptor. Fortunately the results allowed us to examine in more detail the formation of 'anomalous' $\alpha_2\text{M}$ -trypsin complexes previously described (Van Leuven *et al.*, 1982).

Experimental

Materials

Iodoacetamide, *p*-chloromercuribenzoate and *N*-ethylmaleimide were from Sigma. Nph_2SCN and Nbs_2 were from Aldrich. Amersham International supplied Na^{125}I (carrier-free), iodo[1- ^{14}C]acetamide (sp. radioactivity 53 mCi/mmol), [^{14}C]methylamine (sp. radioactivity 56 mCi/mmol) and [1,3- ^3H]iPr $_2$ P-F (sp. radioactivity 6.5 Ci/mmol). Tos-Phe- CH_2Cl -treated trypsin was from Worthington.

Methods

$\alpha_2\text{M}$ was isolated from human citrated plasma as described previously (Van Leuven *et al.*, 1980). Labelling with Na^{125}I and chloramine-T, cell culture of normal human skin fibroblasts and measurements of receptor-mediated endocytosis were also as described previously (Van Leuven *et al.*, 1979, 1980).

Rate electrophoresis. Electrophoretic mobility of native proteins was examined by rate electrophoresis in 5% (w/v)-acrylamide gels. A discontinuous buffer system was used. The reservoir buffer was 0.041 M-Tris/0.040 M-borate, pH 8.6; the stacking gel was 4% acrylamide in 0.054 M-Tris/0.020 M- H_2SO_4 , pH 6.1; the separating gel was 5% acrylamide in 0.095 M-Tris/HCl, pH 5.7. Samples were diluted in reservoir buffer and contained 10% (v/v) glycerol. Gels were cast in cassettes (Pharmacia, Uppsala, Sweden) of dimensions 8 cm $\times$ 8 cm $\times$ 0.27 cm and run for 3 h at 125 V. Gels were subsequently fixed in 10% (w/v) trichloroacetic acid, stained with Coomassie Brilliant Blue (at 60°C) and destained by diffusion overnight. Photographs were taken with a Polaroid camera using positive/negative type 665 film. Quantification of radioactivity was done by excision of Coomassie Blue-stained bands with a razor blade. The pieces of gel were dissolved in 1 ml

of aq. 30% (v/v) H_2O_2 at 56°C overnight. After addition of 15 ml of scintillation cocktail (Instagel II; Packard) radioactivity was counted in a Packard Tri-Carb liquid-scintillation counter. Counting efficiency was determined with an internal standard ([^{14}C]toluene; Packard). This procedure was shown to be accurate for determination of specific radioactivity of radiolabelled proteins (Van Leuven *et al.*, 1981a,b, 1982).

Trypsin activity towards Bz-Arg-pNA. This was measured at 37°C as described previously (Van Leuven *et al.*, 1981a); the assay mixture contained Tris/HCl buffer (0.1 M, pH 8.2), 20 mM- CaCl_2 , 2 mM-Bz-Arg-pNA and enzyme in a final volume of 2.5 ml. A_{410} was continuously recorded. Activities are expressed as $\Delta A_{410}/\text{min}$ at 37°C. When indicated, soya-bean trypsin inhibitor was added, equivalent to the amount of trypsin present on a weight basis.

Proteolytic activity towards [^3H]acetylated haemoglobin was measured. Bovine haemoglobin (type I, Sigma) was labelled with [^3H]acetic anhydride (3 Ci/mmol; Amersham); 600 mg of haemoglobin was allowed to react with 25 mCi of [^3H]acetic anhydride in 0.5 M-borate/1.4 M-sodium acetate buffer, pH 9.0, at 0°C for 24 h. The labelled protein was collected and freed from unchanged anhydride by repeated precipitation with trichloroacetic acid and finally dialysed against saline (0.15 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, the following: Tris/HCl (50 mM, pH 8.2), 100 μl of the labelled haemoglobin preparation and either trypsin (1 μg) or $\alpha_2\text{M}$ (200 μg). Incubation at 37°C for 1 h was followed by addition of 0.5 ml of outdated newborn-calf serum (Gibco) 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 supernatant was added to 15 ml of Instagel II. Radioactivity was counted in a Tri-Carb liquid-scintillation counter with external standardization.

Active-site titration of trypsin in $\alpha_2\text{M}$ -trypsin complexes was done with [^3H]iPr $_2$ P-F; the protein was made to react with [^3H]iPr $_2$ P-F (0.77 mM) in 50 mM-Tris/HCl, pH 8.0 for 1 h. After separation by rate electrophoresis, radioactivity was determined as described above.

Determination of thiol residues. Thiol residues on $\alpha_2\text{M}$ were determined either spectrophotometrically with Nbs_2 essentially as described by Sottrup-Jensen *et al.* (1980) or by derivatization with iodo[^{14}C]acetamide. For the latter, $\alpha_2\text{M}$ -complexes were made to react with 0.5 mM-iodo[^{14}C]acetamide for 2 h or for the time periods indicated (Table 1). After separation by rate electrophoresis, radioactivity was determined as described above.

Results and discussion

Thiol residues on α_2 M complexes

To determine the exposed thiol (cysteine) residues on α_2 M–proteinase and on α_2 M–methylamine complexes, the complexes were carboxymethylated with iodo[14 C]acetamide. Radioactivity in α_2 M was determined after separation by rate electrophoresis on 5% polyacrylamide gels (Van Leuven *et al.*, 1981a). Negligible incorporation was found in native α_2 M (Table 1). On complexes prepared either with trypsin or with methylamine, apparently two thiol groups reacted (Table 1). As four were expected (one per monomer), thiol residues were therefore also determined spectrophotometrically with Nbs₂ essentially as described by Sottrup-Jensen *et al.* (1980). By this procedure, four thiol groups were measured, at least when the α_2 M–trypsin complexes were measured immediately (Table 1). When the α_2 M–trypsin complexes were left for 30 min at room temperature before reaction with Nbs₂, only about half of the original thiol

groups could be detected (Table 1). This indicates that two out of the four thiol groups on α_2 M–trypsin complexes are rapidly oxidized under these conditions, corroborating the results obtained with iodo[14 C]acetamide. With α_2 M–methylamine complexes also, only two thiol groups could be detected by the Nbs₂ reaction (Table 1).

To circumvent the rapid oxidation of thiol groups, the reaction with methylamine was done with iodo[14 C]acetamide present during incubation with methylamine. Subsequent separation by rate electrophoresis showed that, under these conditions, treatment with methylamine did result only partially in fast-form α_2 M (Fig. 1), although quantification of label indicated that nearly four thiol groups were exposed (Table 1).

Other thiol-specific reagents were tested under similar conditions. Nbs₂ (1 mM) and *p*-chloromercuribenzoate (0.1 mM) did not appreciably interfere with the conformational change (generation of fast-form α_2 M) by treatment with methylamine (Fig. 1). *N*-Ethylmaleimide was about equally effective as

Table 1. Determination of thiol residues on α_2 M complexes

Quantification of thiol residues was performed by reaction with iodo[14 C]acetamide and separation by rate electrophoresis or spectrophotometrically with Nbs₂ (see under 'Methods'). In Expt. (A) α_2 M–trypsin complexes were prepared by reaction of a 3:1 molar excess of trypsin (Tos-Phe-CH₂Cl-treated, 65% active) with α_2 M. Excess trypsin was removed by gel filtration. The α_2 M–methylamine derivative was obtained by reaction of α_2 M with 0.2 M-methylamine at 25°C in Tris/HCl (50 mM, pH 8.0) for 6 h. In Expt. (B) the same conditions were used to prepare the α_2 M–trypsin complex, but determination was done either immediately (2 min) or after the complexes were left for 30 min at room temperature. Excess trypsin was not removed, as the enzyme preparation was found not to contain free thiol groups. In Expt. (C) α_2 M was allowed to react with 25 mM-methylamine (Tris/HCl, 50 mM, pH 8.0, 25°C) but overnight and in the presence of 0.5 mM-iodo[14 C]acetamide. Results are the means of two or three determinations.

Expt.	Treatment	Thiol groups (mol/mol of α_2 M) reactive with:	
		iodo[14 C]acetamide	Nbs ₂
(A)	α_2 M	0.2	0.3
	α_2 M–trypsin	1.90	1.75
	α_2 M–methylamine	1.84	1.68
(B)	α_2 M–trypsin		
	2 min	—	4.10
	30 min	—	2.25
(C)	α_2 M–methylamine	3.76	—

Fig. 1. Effect of thiol-specific reagents on conformational change of α_2 M by reaction with methylamine

α_2 M (2 mg/ml) was allowed to react with 25 mM-methylamine in Tris/HCl (50 mM, pH 8.0) at 25°C overnight. The reaction mixture contained no further additions (1) or 1 mM-iodoacetamide (2), 1 mM-*N*-ethylmaleimide (3), 1 mM-Nbs₂ (4), 1 mM-Nph₂SCN (5) or 0.1 mM-*p*-chloromercuribenzoate (6). Rate electrophoresis was done on 5% polyacrylamide gels and 10 μ g of protein was loaded per lane. Slow- and fast-form α_2 M and the position of the dye front are indicated on the right-hand side.

iodoacetamide (both at 1 mM). The most powerful agent was Nph_2SCN : practically no fast-form $\alpha_2\text{M}$ was generated when Nph_2SCN was present during methylamine treatment (Fig. 1). Incorporation of [^{14}C]methylamine was not inhibited, neither by iodoacetamide nor by Nph_2SCN ; only the distribution of label in slow- and fast-form $\alpha_2\text{M}$ was

affected (Table 2). The difference between these thiol-reactive probes might be related to the rate at which they react or to steric factors.

Nph_2SCN , which has been shown to act as a specific cyanylating agent for thiol groups (Degani & Degani, 1980), introduces the least bulky group on to $\alpha_2\text{M}$. This in itself can influence the rate of reaction with the thiol group, but apparently does not affect the change in conformation, since four thiol groups can react with Nbs_2 on $\alpha_2\text{M}$ -complexes (Table 1), whereas Nbs_2 is the least effective in blocking the conformational change (Fig. 1). Further experimental work will have to be done to reveal whether the microenvironments of these thiol groups on $\alpha_2\text{M}$ complexes are different. This would add another argument to our previous findings indicating that the subunits in $\alpha_2\text{M}$ are asymmetrically organized in the tetramer (Van Leuven *et al.*, 1981a,b).

Soya-bean-trypsin-inhibitor-inhibited complexes

Apart from these considerations, the present findings allowed us to examine further the mechanism of conformational change by which $\alpha_2\text{M}$ inhibits proteinases. As Nph_2SCN did not inhibit [^{14}C]methylamine incorporation but affected only the distribution of label between slow- and fast-form $\alpha_2\text{M}$ (Table 2), this was reminiscent of our previous findings that [^{14}C]methylamine incorporation proceeds via labelled slow-form $\alpha_2\text{M}$, which to some extent proved stable to isolation by polyacrylamide-gel electrophoresis (Van Leuven *et al.*,

Table 2. Effect of iodoacetamide and Nph_2SCN on [^{14}C]methylamine incorporation into $\alpha_2\text{M}$

$\alpha_2\text{M}$ was allowed to react with [^{14}C]methylamine (25 mM) in Tris/HCl (50 mM, pH 8.0) at 25°C for 3 h, without further additions, or in the presence of 1 mM-iodoacetamide or 1 mM- Nph_2SCN . After rate electrophoresis (see Fig. 1), slow- and fast-form $\alpha_2\text{M}$ were excised separately from the gels and radioactivity determined in both. Total incorporation is the mathematical sum of these two determinations. Although after 3 h incubation methylamine incorporation is not complete, this experiment is representative for the extent of incorporation of [^{14}C]methylamine into the two forms of $\alpha_2\text{M}$ for longer periods of incubation.

Additions	[^{14}C]Methylamine incorporation (c.p.m./10 μg) into:		
	Slow form	Fast form	Total
None	468	2735	3203
Iodoacetamide (1 mM)	1737	1385	3122
Nph_2SCN (1 mM)	2743	591	3334

Table 3. Enzymic activity of trypsin and $\alpha_2\text{M}$ -trypsin complexes

Enzymic activity towards [^3H]acetylated haemoglobin and Bz-Arg-pNA was measured as detailed in the Experimental section. Complexes were prepared and isolated as described in the text. In the haemoglobin assay, activity is expressed as radioactivity (c.p.m.) not precipitated by trichloroacetic acid, liberated by 1 μg of trypsin or by the equivalent of 200 μg of $\alpha_2\text{M}$ as trypsin complexes. In the Bz-Arg-pNA assay, activity is expressed as increase in absorbance at 410 nm/min with 10 μg of trypsin or the equivalent of 125 μg of $\alpha_2\text{M}$ as trypsin complexes. Tos-Lys- CH_2Cl was used at a final concentration of 2 mM; 20 μg of soya-bean trypsin inhibitor (STI) was used per assay. Results are means of duplicate determinations. Results from the haemoglobin assay are corrected for a blank value of 1169 c.p.m. (no trypsin or $\alpha_2\text{M}$ added, but otherwise identical conditions).

Conditions	Substrate	Enzymic activity	
		Bz-Arg-pNA ($\Delta A/\text{min}$)	[^3H]Haemoglobin (c.p.m.)
$\alpha_2\text{M}$ (native)	...	0.001	22
Trypsin		0.041	10417
+ STI		0.001	22
+ Tos-Lys- CH_2Cl		0.001	1234
$\alpha_2\text{M}$ -trypsin		0.030	3266
+ STI		0.028	1862
+ Tos-Lys- CH_2Cl		0.002	1259
$\alpha_2\text{M.MA.CN}$ -trypsin		0.015	9825
+ STI		0.002	1511
+ Tos-Lys- CH_2Cl		0.001	1036

1981a). We hypothesized that the presence of this form might have led to our finding of 'abnormal' α_2 M-trypsin complexes that were proteolytically active and were inhibited by soya-bean trypsin inhibitor (Van Leuven *et al.* 1981b).

We prepared several batches of α_2 M.MA.CN as follows. α_2 M (10mg) was allowed to react with methylamine (25mM) in the presence of Nph_2SCN (1mM) at pH 8.0 (Tris/HCl, 50mM) at room temperature overnight and the derivative was isolated by gel filtration (Ultrogel AcA-22; LKB). Control preparations of α_2 M.MA (no Nph_2SCN present) were obtained under otherwise identical conditions. Rate electrophoresis showed α_2 M.MA.CN to be about 90% slow form, whereas α_2 M.MA contained about 90% fast form (judged by visual inspection of the gels).

Native α_2 M, α_2 M.MA and α_2 M.MA.CN were made to react with a 3:1 molar excess of trypsin (65% active trypsin as determined by active-site titration). On rate electrophoresis, all preparations were shown to be transformed into fast-form α_2 M. Thus trypsin overrides the block in conformational change imposed by derivatization of the thiol groups in α_2 M.MA.CN. The α_2 M complexes were subsequently separated from excess trypsin by gel filtration and examined for trypsin activity (Table 3).

Amidase activity, measured spectrophotometrically towards Bz-Arg-pNA, showed less than 10% of the total activity of α_2 M-trypsin to be inhibited by soya-bean trypsin inhibitor, whereas Tos-Lys- CH_2Cl inhibited completely. This is the classical behaviour of α_2 M-proteinase complexes. An equivalent amount of α_2 M.MA.CN-trypsin complexes showed only 50% of the activity of the control preparation, and this activity was completely inhibited by soya-bean trypsin inhibitor. Under the same conditions, α_2 M.MA (fast form) did bind less than 10% of the amount of trypsin bound by native α_2 M.

Proteolytic activity was measured towards

[^3H]acetylated haemoglobin as previously described (Van Leuven *et al.*, 1981b). Although some activity was found associated with α_2 M-trypsin complexes, the proteolytic activity of α_2 M.MA.CN-trypsin was much higher (Table 3). All activity was inhibited by soya-bean trypsin inhibitor.

As a third approach we measured the incorporation of [^3H]iPr $_2$ P-F into the complexes; radioactivity was quantified after separation by rate electrophoresis. Relative to α_2 M-trypsin, α_2 M.MA.CN-trypsin complexes incorporated about 45% of [^3H]iPr $_2$ P-F, whereas incorporation in native α_2 M was negligible (Table 4).

From these findings it is clear that trypsin complexes are formed with α_2 M.MA.CN, as judged from the incorporation of [^3H]iPr $_2$ P-F. However, only about half as much trypsin forms a complex with α_2 M.MA.CN as compared with α_2 M, as shown by both amidase activity towards Bz-Arg-pNA (Table 3) and by active-site labelling with [^3H]iPr $_2$ P-F (Table 4). Moreover, essentially all trypsin bound to α_2 M.MA.CN is inhibited by soya-bean trypsin inhibitor, concomitant with a high proteolytic activity in the haemoglobin assay.

These data strengthened our previous hypothesis, namely that slow-form α_2 M, in which the thioester is destroyed by primary amines, can be generated (Van Leuven *et al.*, 1981a). The stability of this form is increased when the cysteine thiol groups are derivatized, especially by cyanylation. However, trypsin complexes derived from this form of α_2 M contain only half as much proteinase as normal complexes and, more importantly, must be designated 'abnormal' or 'open' inasmuch that soya-bean trypsin inhibitor inhibits all the trypsin and that the complexes are proteolytically active (Van Leuven *et al.*, 1982).

Receptor-mediated endocytosis and binding of monoclonal antibody (F2B2)

As stated previously (Van Leuven *et al.*, 1982), it seemed worthwhile to determine whether generation of 'abnormal' complexes would have biological implications. Therefore we examined the behaviour of the 'open' complexes in receptor-mediated endocytosis by the cellular assay (Van Leuven *et al.*, 1979, 1980) and the receptor recognition site on the α_2 M complexes, with the monoclonal antibody, F2B2. The latter was shown to be totally specific for α_2 M complexes generated either by proteinase or by methylamine treatment (Marynen *et al.*, 1981). Moreover, this antibody, and Fab portions derived from it, blocked the receptor-recognition site on the α_2 M complexes (Marynen *et al.*, 1981). The binding of F2B2 to α_2 M complexes is fast and stable and can be detected by rate electrophoresis on 5% polyacrylamide gels (Marynen *et al.*, 1981). This procedure was applied to the complexes generated in

Table 4. [^3H]iPr $_2$ P-F incorporation in α_2 M-trypsin complexes

α_2 M derivatives and complexes with trypsin were prepared as described in the text. They were made to react with [^3H]iPr $_2$ P-F (0.77mM) for 1h. After separation by rate electrophoresis, radioactivity in α_2 M was determined (see under 'Methods'). In this case counting efficiency was not determined, and the results are given in c.p.m./10 μg of α_2 M.

	Incorporation	
	c.p.m./10 μg	Relative (%)
α_2 M-trypsin	17234	100
α_2 M.MA.CN-trypsin	7747	45
α_2 M	510	3

Fig. 2. Binding of the monoclonal antibody (F2B2) to α_2 M-derivatives

Derivatives of α_2 M (20 μ g), prepared as described in the text, were allowed to react with the monoclonal antibody F2B2 (20 μ g of protein) for 30 min at 25°C. Rate electrophoresis of 5% polyacrylamide gels was carried out on these and the untreated derivatives. (1) Native α_2 M; (2) α_2 M.MA.CN-trypsin; (3) α_2 M.MA; (4) α_2 M.MA.CN; in each case untreated (—) or treated with F2B2 (+). The position of slow- and fast-form α_2 M, of the free antibody (F2B2) and the dye front are marked. Note the absence of reaction with native α_2 M (Marynen *et al.*, 1981) and the shift in mobility of the band when reaction has occurred with other derivatives. Some slow-form α_2 M, still present in the α_2 M.MA (3) (Van Leuven *et al.*, 1981a) has also reacted with F2B2, indicating that the thioesters were disrupted.

the experiments described above. As judged from Fig. 2, binding of the monoclonal antibody to the anomalous complexes was of the normal extent; the pattern of bands obtained was identical with that obtained with classical α_2 M-trypsin complexes (Marynen *et al.*, 1981). Surprisingly, however, whereas F2B2 did not react with native α_2 M, slow-form α_2 M.MA.CN did bind the monoclonal antibody (Fig. 2, lane 4).

We have previously shown that fibroblasts in culture carry a receptor at their plasma membrane which discriminates α_2 M complexes from native α_2 M; as a consequence, only α_2 M-proteinase complexes are internalized by receptor-mediated endocytosis (Van Leuven *et al.*, 1978, 1979; Marynen *et al.*, 1981). To examine whether the receptor would also recognize the anomalous complexes described, we measured inhibition of endocytosis of 125 I-labelled α_2 M-trypsin complexes by the derivatives obtained. It was found that α_2 M.MA.CN-trypsin complexes were nearly as potent as α_2 M-trypsin in inhibiting endocytosis, indicating their effective recognition by the receptor. Moreover, slow-form α_2 M.MA.CN was also inhibitory for endocytosis, whereas native α_2 M was not (Table 5), confirming our finding with the monoclonal antibody described above.

Taken together, these results indicate that, despite the proteolytic nature of the anomalous complexes, their interactions with the highly specific probes (cellular receptor and monoclonal antibody) are not impaired or even markedly altered. The data further indicate that cyanilation or carboxymethylation of the cysteine thiol group does not interfere in the two systems mentioned; therefore it does not appear that the thiol groups as such participate in receptor-

Table 5. Receptor-mediated endocytosis of 125 I-labelled α_2 M-trypsin complexes by fibroblasts in culture: inhibition by α_2 M derivatives

Normal human fibroblast monolayers were examined for receptor-mediated endocytosis for 30 min at 37°C with 125 I-labelled α_2 M-trypsin complexes (50 μ g/ml), without further additions or with the derivatives indicated at 200 μ g/ml of medium unless otherwise indicated. Results are expressed relative to control (no addition, 5412 c.p.m. per 10^5 cells endocytosed). General procedures were as described previously (Van Leuven *et al.*, 1980). Derivatives of α_2 M were prepared as described in the text. Results are means of duplicate cell layers in two experiments.

Additions	Receptor-mediated endocytosis relative to control (%)
α_2 M	96
α_2 M-trypsin	36
α_2 M-methylamine	44
α_2 M-trypsin/carboxymethylated	41
α_2 M-methylamine/carboxymethylated	46
α_2 M.MA.CN	
50 μ g/ml	78
200 μ g/ml	49
α_2 M.MA.CN-trypsin	
50 μ g/ml	60
200 μ g/ml	33

mediated endocytosis (Table 5). Thus our results indicate that the structure making up the receptor-recognition site on α_2 M complexes does not include the thioester residues and is not affected by the anomaly of the complex. This is substantiated both by the cellular assay (Van Leuven *et al.*, 1979) and by the normal binding of the complex-specific monoclonal antibody (Marynen *et al.*, 1981). This indicates that if the conformational change of α_2 M in the complex were to be incomplete, so as not to 'wrap' the proteinase completely (as can be envisaged in a normal complex), the difference would be only minute and would not affect the receptor-recognition site on α_2 M complexes. This obviously is advantageous for the situation *in vivo*: even the formation of proteolytically active α_2 M-proteinase complexes will lead to their removal and degradation by cellular mechanisms before they can become harmful in the circulation or in the extracellular space. Apparently this mechanism extends even further. Our data indicate that slow-form α_2 M, in which the active thioesters are destroyed (α_2 M.MA.CN), is also internalized by receptor-mediated endocytosis (Table 5) and binds the monoclonal antibody (Fig. 2). These findings indicate that the receptor-recognition site on α_2 M is already accessible to both the receptor and the monoclonal antibody before the dramatic change in conformation occurs. The only necessary condition for revealing the receptor-recognition site seems to be the disruption of the covalent internal thioester, which is of course expected to produce minor changes in conformation locally. Therefore it seems likely that the receptor-recognition site is located in the vicinity of the internal thioester.

Conclusions

Our data indicate that disruption of the internal thioesters in α_2 M by primary amines is sufficient to expose the receptor recognition site and does not necessarily lead to the conformational change typical of proteinase complexes. Moreover, the sequence of events, namely breaking the thioester, followed by proteinase complex-formation, leads to equimolar complexes instead of the usual 2:1 binding of trypsin to α_2 M. The proteinase in the resulting complex is fully accessible to soya-bean trypsin inhibitor and has proteolytic activity. This might explain the disparate data in the literature on

the exact stoichiometry of proteinase- α_2 M complex-formation and on the proteolytic character of the complexes produced. Clearly such studies require fully active α_2 M, in which activity is best assessed not only by rate electrophoresis but preferably by titration of the active thioesters with [14 C]methylamine. A perfect probe appears the monoclonal antibody F2B2 (Marynen *et al.*, 1981), which distinguishes in slow-form α_2 M, two species that are dependent on the state of the thioesters.

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