TRYPSIN-INDUCED ACTIVATION OF THE THIOL ESTERS IN α_2-MACROGLOBULIN GENERATES A SHORT-LIVED INTERMEDIATE ('NASCENT' α_2M) THAT CAN REACT RAPIDLY TO INCORPORATE NOT ONLY METHYLAMINE OR PUTRESCINE BUT ALSO PROTEINS LACKING PROTEINASE ACTIVITY

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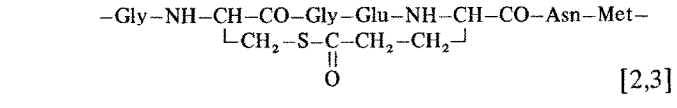
1. Introduction

Each of the 4 identical M_r 180 000 subunits of human α_2-macroglobulin (α_2M) contains a reactive Glx-residue [1], whose γ-carboxyl group is esterified to the sulfhydryl group of the cysteinyl residue in the sequence:



[2,3]

The Glx-residue is located ~469 residues from the C-terminus of α_2M [3].

Complement component C3 also contains a reactive thiol ester [4,5], located in an identical heptapeptide sequence in its α'-chain [6]. Following limited proteolysis in the 'bait' region of α_2M [2,3] or at that site in C3 which generates C3a and C3b [4–6] the thiol esters are rapidly cleaved resulting in the appearance of free sulfhydryl groups [2–6].

Reaction of α_2M and C3 with methylamine leads to relatively slow cleavage of their thiol esters [2,4–6]. During this process a stoichiometric amount of CH_3NH_2 is incorporated into the reactive Glx-residue to form γ-glutamylmethylamide [1–4,6,7]. As a result of this reaction the proteinase binding capacity of α_2M [1,2,8] and the hemolytic activity of C3 [4–6] are lost, indicating that the thiol-esterified Glx-residues of the two proteins may participate in 'acyl transfer' reactions [2,5,6] which could explain the formation of apparently covalent α_2M–proteinase complexes [9–11] and also the apparently covalent attachment of C3b to cell membranes [6,12,13] and of C4b to IgG [14]. The activated (also known as nascent), form of C3b is said to exist only transiently [15].

Here we show that when the thiol esters in α_2M are activated by the complex formation of α_2M with trypsin, a corresponding short-lived intermediate is generated which can react rapidly and covalently with methylamine or putrescine. We also show that either TLCK–trypsin or insulin, when present during trypsin-induced activation, is incorporated into α_2M.

Abbreviations: α_2M, α_2-macroglobulin; CHOM, chicken ovomucoid; putrescine, 1,4-diaminobutane; TLCK, N^{α}-tosyl-L-lysine chloromethyl-ketone; IgG, immunoglobulin G; PTI, bovine pancreatic trypsin inhibitor (Kunitz); DTT, dithiothreitol

2. Materials and methods

Human α_2M was prepared by Zn^{2+}-affinity chromatography as in [2]. Bovine trypsin (60% active) (Boehringer, Mannheim) was purified by affinity chromatography using a column of chicken ovomucoid (Boehringer) coupled to Sepharose 4B (Pharmacia, Uppsala) as in [16]. The resulting trypsin preparation was 88% active. TLCK–trypsin [17] was prepared from ^{125}I labelled [18] or unlabelled trypsin and was freeze-dried after filtration on Sephadex G-25 in 0.1 M NH_4HCO_3. Porcine insulin (Nordisk Insulin, Gentofte) and insulin specifically mono-iodinated [19] with ^{125}I on Tyr-14 of its A-chain (1.5 Ci/μmol) were gifts from J. Gliemann and O. Sonne, University of Aarhus. PTI was obtained from Novo (Bagsværd). The concentration of α_2M was determined using

$E^{1\%}_{280}$ = 9.1 [20] and M_r 725 000 [21]. The concentration of TLCK-trypsin was determined using $E^{1\%}_{280}$ = 15.4 and M_r 24 000 [22]. The concentration of insulin was determined by amino acid analysis. [^{14}C]Methylamine was from New England Nuclear (Boston MA) (48 Ci/mol) or the Radiochemical Centre (Amersham) (53 Ci/mol). [1,4-^{14}C]Putrescine (122 Ci/mol) was from Amersham. Methylamine · HCl and putrescine · 2 HCl was from Sigma (St Louis MO). A stock solution of α_2M (15.0 μM) in 0.05 M Na-phosphate, 0.1 M NaCl (pH 8.0) was used. A stock solution of trypsin was in 1 mM HCl (CHOM-purified trypsin, active site titre 183 μM). Solutions of TLCK–trypsin (133 μM) and TLCK–^{125}I-trypsin (133 μM) were in 0.1 M Na-phosphate (pH 8.0) (buffer A). Solutions of 0.05–1.0 M radiolabelled methylamine and putrescine were in buffer A. The extent of incorporation of ^{14}C-labelled methylamine and putrescine into α_2M was measured by precipitating α_2M with 10% trichloroacetic acid on discs of Whatman 3 MM filter paper and removing free amine with 5% trichloroacetic acid and ethanol/acetone (1:1, v/v) [2]. Since the denaturation of α_2M with trichloroacetic acid also results in complete cleavage of any remaining unreacted thiol ester we found it necessary to include NH_3 as a 'competing' nucleophile in this assay [2].

3. Results and discussion

At <1 mol trypsin/mol α_2M, 4 mol SH appear/mol trypsin added. Maximally 4 mol SH appear at >2 mol trypsin/mol α_2M [2].

To investigate if activation of the thiol ester of α_2M by proteinases generate an 'acyl transfer' potential as postulated [2,5,6] α_2M was activated by incubating for 1 min with increasing concentrations of trypsin in the presence of [^{14}C]methylamine or [^{14}C]putrescine. Fig.1 shows that the extent of covalent incorporation of these two amines into α_2M parallels the trypsin-induced appearance of SH-groups [2]. Thus, the results show that α_2M when activated by proteinases can react rapidly to covalently incorporate primary amines. Despite the inclusion of NH_3 in the assay mixture the values of methylamine and of putrescine incorporated into α_2M in the absence of trypsin were found to be 0.40 and 0.55 mol/mol α_2M, respectively. The incorporation of these amines at 25 mM in the presence of 1 mol trypsin/mol α_2M was 2.4–2.5 mol/mol α_2M; and 2.7–2.9 mol/mol α_2M

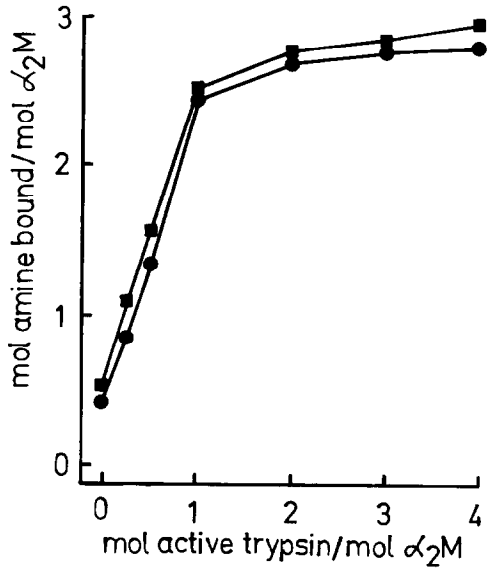


Fig.1. Incorporation of CH_3NH_2 and putrescine into α_2M at different trypsin concentrations. For each point 10 μl α_2M (15.0 μM) was mixed with 5 μl CHOM-purified trypsin (diluted from 120 μM) and 10 μl CH_3NH_2 or putrescine (62.5 mM). After incubation for 60 s at room temperature 25 μl 1 M NH_4HCO_3 was added. [α_2M] = 6.0 μM; [trypsin] = 1.5–24 μM; [CH_3NH_2] or [putrescine] = 25 mM. A 40 μl aliquot was spotted on a filter disc and the α_2M precipitated with 10% trichloroacetic acid as in section 2. CH_3NH_2, (•); putrescine (▪).

at 4 mol trypsin/mol α_2M. The amount of CH_3NH_2 bound at 100 mM CH_3NH_2 was ~3.0 mol/mol α_2M at 2 mol trypsin/mol α_2M. At 0.44 mM CH_3NH_2 0.3 mol/mol α_2M was bound, showing that covalent binding may occur even at this, much lower, concentration of the nucleophile. Thus, the proteinase-induced activation of the α_2M-thiol esters apparently makes the reactive Glx-residues, which are normally available only to slow covalent incorporation of small primary amines, e.g., CH_3NH_2 [1–3], accessible to fast covalent incorporation not only of CH_3NH_2 but also of the larger putrescine. This ability for fast covalent incorporation of amines turned out to be a fleeting activity of the trypsin-activated α_2M which decreases rapidly as shown in fig.2, where α_2M was first activated with trypsin (2 mol/mol α_2M) and then, after a variable delay of 5–120 s, incubated with CH_3NH_2 for 60 s. However, it is also evident from fig.2 that even after 120 s as much as 0.5 mol CH_3NH_2/mol α_2M could still be bound covalently.

In separate experiments we found that at 2 mol trypsin bound/mol α_2M (in the absence of CH_3NH_2)

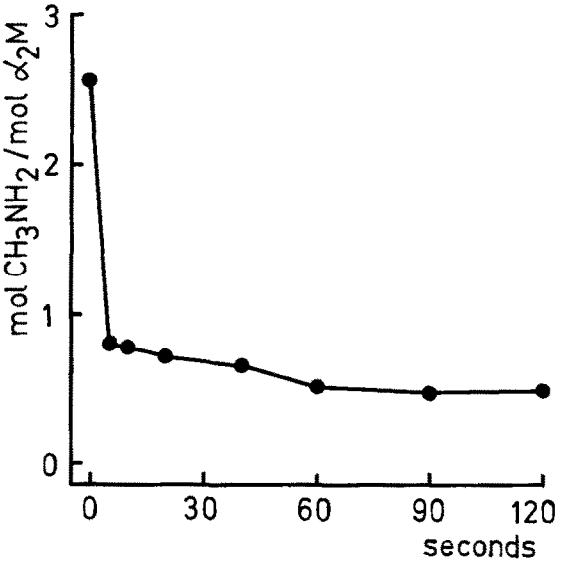


Fig.2. Incorporation of CH_3NH_2 into α_2M which had been activated with trypsin before adding the CH_3NH_2. To 20 μl α_2M (15.0 μM) 10 μl CHOM-purified trypsin (60 μM) was added, followed by addition of 20 μl $^{14}CH_3NH_2$ (62.5 mM) at a certain time interval after trypsin addition. [trypsin] = 12.0 μM; [α_2M] = 6.0 μM; [CH_3NH_2] = 25 mM. The mixtures were then incubated for 60 s after which 40 μl 1 M NH_4HCO_3 was added. Then an 80 μl aliquot was spotted on a filter disc and the α_2M precipitated with 10% trichloroacetic acid as in section 2.

~1.3 mol trypsin was bound 'covalently' in the sense that it was not separated from α_2M when the DTT-treated, carboxymethylated complex was gel-filtered in 6 M guanidine–HCl at pH 8.0 [18]. When CH_3NH_2 was present during trypsin-induced activation of α_2M, the total amount of trypsin bound was still the same 2 mol/mol α_2M but now only 0.6 mol was bound covalently by the same criterion [18]. Those results can be rationalized by proposing that on trypsin-induced activation the thiol esters of α_2M are cleaved to produce a highly reactive, short-lived intermediate, which will react with available nucleophiles, e.g., proteinase, methylamine, putrescine and probably water, leading to covalent incorporation of the respective nucleophile on the γ-carbonyl group of the reactive Glx-residue. This short-lived α_2M-intermediate is apparently the functional equivalent of the nascent C3b, as shown [23] to incorporate not only methylamine but also phenylhydrazine, glucosamine and glycerol. It may also be equivalent with the activated α′-chain of C4b [14] which binds IgG.

To see if proteins other than active proteinases can be incorporated into 'nascent' α_2M, the experiments shown in fig.3 (TLCK–trypsin) and fig.4 (insulin) were done. When present during proteinase-induced (trypsin) activation of α_2M 0.45 mol TLCK–trypsin/mol α_2M was incorporated (0.26 mol covalently by the gel-filtration criterion) for 2.14 mol active trypsin/mol α_2M (fig.3). In the insulin case (fig.4) up to 0.32 mol insulin/mol α_2M was incorporated (0.11 mol covalently) for 1.61 mol active trypsin/mol α_2M. In the absence of active trypsin no incorporation of either protein was observed. Furthermore, when added 2 min after the active trypsin, only 0.07 mol TLCK–trypsin/mol α_2M was bound. Thus, TLCK–trypsin and insulin, both proteins lacking proteinase activity, can be incorporated into α_2M, but only by its proteinase-activated 'nascent' form.

Earlier results, e.g. [24], indicating that α_2M could bind insulin can now be explained as having been due to ongoing partial activation to 'nascent' α_2M by active proteinases that must have been present because serum, not plasma, was used as a source of α_2M.

Considering these results it becomes important to define the extent of proteolytic activation and thiol-

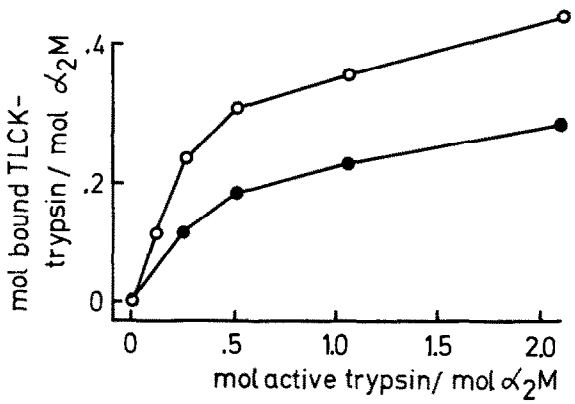


Fig.3. Binding of TLCK–^{125}I-trypsin to α_2M at different concentrations of active trypsin. Each 500 μl sample of α_2M containing a 1.77-fold molar excess of TLCK–^{125}I-trypsin was incubated with active trypsin for 2 min. Trypsin action was stopped by adding 100 μl PTI (1.54 mM). All samples were then subjected to gel chromatography in buffer A (○) on a column of Sephacryl S-300 (1 × 94 cm; 4.0 ml/h; 1.0 ml fractions). The material from each sample, eluting in the position of α_2M (see fig.1 of [18]) was pooled, precipitated with trichloroacetic acid (10%, w/v), collected, washed with ether, redissolved in 1.0 ml buffer B (0.5 M Tris–HCl, 6 M guanidine–HCl, pH 8.0), reduced (20 μl 0.5 M DTT, 1 h) alkylated (50 μl 0.5 M ICH_2CONH_2 10 min) then rechromatographed in buffer B on Sephacryl S-300 (1 × 93 cm; 4.0 ml/h; 1.3 ml fractions). (●) Represents TLCK–^{125}I-trypsin remaining bound to α_2M fragments (eluting in tubes 23–33; see fig.2 of [18]), under these denaturing conditions.

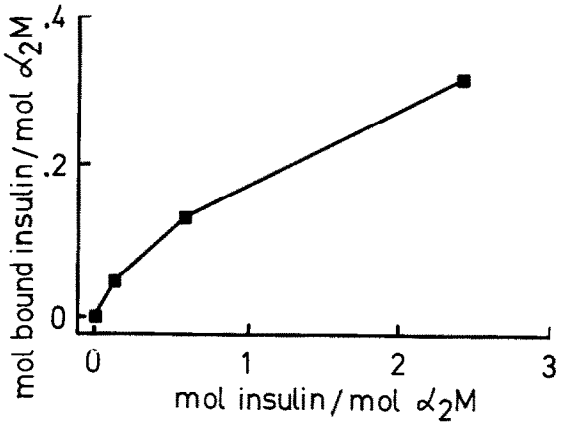


Fig.4. Binding of ^{125}I-labelled insulin to α_2M at different concentrations of insulin. Samples (500 μl) of α_2M containing different amounts of ^{125}I-labelled insulin and unlabelled insulin were incubated with active trypsin at a 1.61-fold molar excess over α_2M for 2 min. Following addition of PTI the samples were gel chromatographed in buffer A. A single experiment performed with a reduced, alkylated sample in buffer B showed that 0.11 mol insulin A-chain/mol α_2M was covalently attached to α_2M for 2.3 mol insulin added/mol α_2M. The conditions for gel chromatography were as in fig.3.

ester cleavage in α_2M preparations used in biological experiments, particularly studies of cellular uptake mechanisms.

These results further stress the functional relationship between α_2M and C3 recognized in [1–3,5,6]. In particular it is striking that both proteins following proteolytic activation interact with their respective cell surface receptors, resulting in endocytosis of α_2M–proteinase complexes [25–27] or opsonisation of complexes between C3b and cells [28–30].

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