A THIOL-ESTER IN α_2-MACROGLOBULIN CLEAVED DURING PROTEINASE COMPLEX FORMATION

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Received 13 October 1980

1. Introduction

The glycoprotein α_2-macroglobulin (α_2M), M_r 725 000, is unique among plasma proteins in being able to form complexes with proteinases from all 4 classes (EC 3.4.21–24) [1–3]. The function of α_2M is not well understood. However, the rapid clearance of α_2M-proteinase complexes from the blood by the reticuloendothelial system [4] and the uptake of α_2M or α_2M-proteinase complexes via receptors by several types of cell in culture [5–8] indicates that α_2M plays a role in the transport of proteinases and possibly other proteins into cells.

The binding of proteinases to α_2M is initiated by cleavage of one or more of the M_r 180 000 subunits of α_2M in the 'bait region' [9], producing two M_r 85 000 fragments [1,10–12]. There appears to be a close-fitting binding site in this area since even anhydrotrypsin binds to α_2M [13]. The exact position of cleavage in the 'bait region' reflects the known substrate specificity of the particular proteinase being complexed (trypsin, plasmin, thrombin, elastase). Following cleavage, the initial complex, involving the proteinase active site and the α_2M 'bait region', is rearranged to form a final complex, involving a second site in the proteinase and probably also in α_2M (L. S.-J., T. E. P., S. M., H. Jörnvall, in preparation). In the final complex α_2M may well be covalently linked to the proteinase [9,14]. The subunits of α_2M can be cleaved specifically by heating, into two fragments of M_r 120 000 and M_r 60 000 [15–17]. This polypeptide chain cleavage occurs at the α-amino group of a particular Glx-residue [16], whose γ-carboxyl group is also the site of covalent incorporation of methylamine [18] during 'inactivation' of the proteinase binding capacity of α_2M [19,20]. The latter two processes are apparently mutually exclusive [16,18].

The complement components C3, 4 and 5 are also inactivated by aliphatic amines [19,21]. The recent demonstration that CH_3NH_2 can react covalently with a Glx-residue in C3 [22] indicates that α_2M, C3, C4 and C5 are structurally and functionally related.

This report concerns a previously unrecognized appearance of thiol groups in α_2M (max. 4 mol/mol α_2M) accompanying complex formation with proteinases. This -SH- appearance is also observed when α_2M is 'inactivated' by CH_3NH_2 or by denaturation. The implications of this for the mechanism of α_2M are discussed and we conclude that the CH_3NH_2-reactive Glx-residue [18] is in fact bound as a γ-glutamyl thiolester to the SH of a cysteinyl residue.

2. Materials and methods

Human α_2M was prepared by Zn^{2+}–chelate affinity chromatography [23] using a slight modification of a method in [24] as summarized: Following removal of plasminogen [25] and precipitation of plasma with polyethylene glycol (av. M_r 4000, 4–12%, w/v) the precipitate was redissolved in 20 mM phosphate buffer (pH 6.4) and dialyzed against running tap-water (11°C). After removal of euglobulins by centrifugation α_2M was adsorbed to Zn^{2+}–Sepharose 4B. Following extensive washing with phosphate (pH 6.4)/0.5 M NaCl α_2M was eluted with 0.1 M Na_2EDTA (pH 7.0). After gel filtration on Sephacryl S-300 in

Abbreviations: α_2M, α_2-macroglobulin; STI, soybean trypsin inhibitor; TPCK, N^{α}-tosyl phenylalanine chloro-methyl ketone; DTNB, 5,5'-dithiobis-2-nitro benzoic acid; *p*-NPGB, 4'-nitrophenyl-4-guanidinobenzoic acid; SDS, sodium dodecyl sulfate

0.05 M Na-phosphate, 0.1 M NaCl (pH 8.0) the resulting α_2M preparations were found to protect 1.7–2.0 mol trypsin/mol α_2M from inhibition by STI, when assayed by active site titration using *p*-NPGB [26,27]. The solutions of α_2M were concentrated to 9–12 mg/ml by ultrafiltration and stored at −20°C. TPCK treated bovine trypsin (active site titre 52%) and STI were obtained from Worthington (Freehold NJ) and porcine pancreatic elastase (assumed to contain ~50% active enzyme) was a gift from D. Shotton (Cambridge). DTNB was obtained from Fluka (Buchs). *p*-NPGB was from Merck (Darmstadt). Bovine trypsinogen and $CH_3NH_2 \cdot HCl$ were from Sigma (St Louis MO). $^{14}CH_3NH_2 \cdot HCl$ was from New England Nuclear (Boston MA) (48 Ci/mol). The buffer used for all incubations was made from Na_2HPO_4 (0.1 M), Na_2EDTA (2 mM) adjusted to pH 8.0 with HCl. The stock solution of α_2M used in all experiments was 13.3 μM. Trypsin was dissolved in 1 mM HCl (active site 107.8 μM) and kept at room temperature. Elastase was dissolved in the phosphate buffer (active site ~105 μM) and kept at 0°C before use. Stock solutions of 0.1 M $CH_3NH_2 \cdot HCl$ and 6 M guanidinium chloride were prepared in the phosphate buffer and the pH readjusted to 8.0 with NaOH. All absorbance measurements were performed in a Beckman DB-24 spectrophotometer at room temperature (22–23°C). The concentration of α_2M was determined using $E^{1\%}_{280} = 9.1$ [28] and $M_r = 725\,000$ [29]. [Thiol] were determined using $\epsilon_{410} = 13\,600\ M^{-1} \cdot cm^{-1}$ at pH 8.0 [30]. A value of $\epsilon_{405} = 16\,240\ M^{-1} \cdot cm^{-1}$ for *p*-nitrophenol was used [31]. ^{14}C was counted in a Beckman LS-330 scintillation counter using 0.24% 2,5-diphenyloxazole (Sigma) in xylene.

3. Results and discussion

When α_2M was incubated with 1 mM DTNB under non-denaturing conditions an increase in A_{410} that remained constant for ≥20 h at room temperature was observed, indicating 0.2 mol thiol/mol native α_2M. Adding sufficient trypsin to saturate α_2M (>2 mol trypsin/mol α_2M) [1,10–12] led to a rapid (5–10 s) increase in absorbance to a new level which remained constant for ≥2 h. A similar increase in absorbance also occurred if α_2M had been preincubated with trypsin for 1 or 10 min and then added to DTNB solution. Inhibition of excess trypsin with STI did not affect these results. After α_2M–trypsin complex had first been incubated with 15 mM ICH_2CONH_2 for 10 min adding DTNB did not lead to an increased in A_{410}. This constitutes independent evidence that the groups made available to titration with DTNB as a result of incubating α_2M with trypsin are indeed thiol groups. If trypsinogen (≤4 mol/mol α_2M) was used instead of trypsin, no SH-groups appeared.

Fig.1 shows the results of incubating a fixed [α_2M] with increasing [trypsin] or [elastase], and of active-site titration of the bound trypsin. The maximal level of thiol appearing was 3.74 mol/mol α_2M indicating that each of its four subunits may contribute one SH-

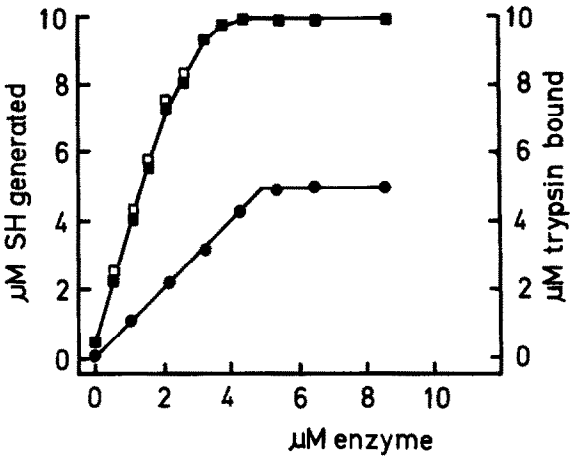

Fig.1. Appearance of thiol-groups in α_2M at varying concentrations of trypsin or elastase, compared with complex formation with trypsin. Thiol generation: To cuvettes containing 700–620 μl phosphate buffer and 200 μl α_2M stock solution was added 0–80 μl trypsin or elastase solution. The reference cuvette contained 900–820 μl phosphate buffer and 0–80 μl proteinase. After mixing by inversion for 15 s the cuvettes were incubated at room temperature for 105 s and the A_{410} adjusted to zero. Then each cuvette received 100 μl 10 mM DTNB in phosphate buffer and the contents were mixed by inversion for 15 s. ΔA_{410} was determined. Release of thiol with trypsin (■), release of thiol with elastase (□). Final concentration of α_2M in the cuvettes was 2.66 μM. Active site titration of bound trypsin: To cuvettes containing 690–610 μl phosphate buffer and 200 μl α_2M solution was added 0–80 μl trypsin solution. The reference cuvette contained 890–810 μl phosphate buffer and 0–80 μl trypsin solution. After mixing by inversion for 15 s the cuvettes were incubated at room temperature for 45 s before 100 μl 227 μM STI in phosphate buffer was added and the contents mixed. After adjusting the A_{405} to zero 10 μl 10 mM *p*-NPGB in dimethylsulfoxide was added and the contents mixed by inversion. The A_{405} was measured at 10 s intervals and extrapolated to the time of mixing. Release of *p*-nitrophenol (●). Final concentration of α_2M in the cuvettes was 2.66 μM.

group. The trypsin-binding capacity was determined as 1.88 mol trypsin/mol α_2M confirming earlier estimates [1,10–12] of 2 mol/mol. On the rising part of the two curves ($\leqslant$2 μM enzyme) 3.7 mol SH appear/mol proteinase added, indicating that even binding of only 1 proteinase molecule suffices to cause the appearance of 4 SH-groups. Under these conditions only two α_2M-subunits were cleaved, as shown in [1,10–12].

Addition of more α_2M to already formed α_2M-trypsin complex (trypsin:α_2M = 1:1 mol/mol) did not cause the appearance of additional thiol groups (not shown). Therefore, we conclude that trypsin in its final complex with α_2M is effectively prevented from reacting with other uncleaved α_2M-molecules. However, the data in fig.2 (results of incubating a fixed [trypsin] with different [α_2M]) indicate that in a large excess of α_2M (3–6.4 μM) an additional generation of up to 3.2 μM thiol was observed above the anticipated level of 8.8 μM. This may be due to a delay between the initial tryptic cleavage of α_2M and the formation of the final α_2M–trypsin complex. Not only incubation with proteinases but also treatment with methylamine leads to the appearance of thiol groups as shown in fig.3. The maximal level of thiol groups appearing is equal to that produced by proteinases. Fig.3 also shows that the relatively slow appearance of thiol on addition of methylamine is accompanied by a corresponding decrease in the trypsin-binding capacity of α_2M. The sum of the thiol groups that have already appeared as a result of methylamine treatment and additional thiol groups that appear immediately when trypsin is added, remains constant.

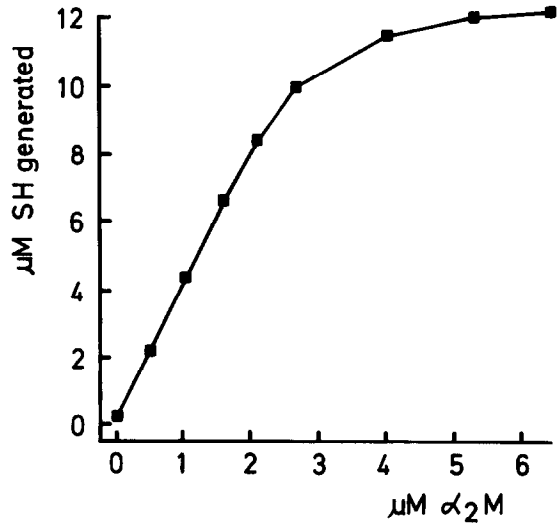

Fig.2. Appearance of thiol groups in α_2M at varying [α_2M]. Trypsin was 4.31 μM. The cuvettes contained 820–360 μl buffer solution, 40 μl trypsin and 40–500 μl α_2M solution. The reference cuvette contained 860 μl phosphate and 40 μl trypsin solution. After incubation for 120 s and adjustment to zero the cuvettes each received 100 μl 10 mM DTNB and ΔA_{410} was measured.

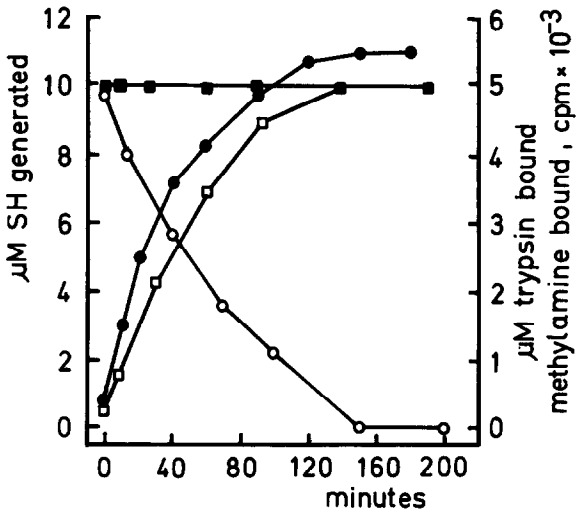

Fig.3. Appearance of thiol in α_2M on incubation with methylamine (50 mM). α_2M solution (4.0 ml) was mixed with 4.0 ml 0.1 M CH_3NH_2 in phosphate buffer (mixture A). Assay of thiols: 400 μl aliquots were removed at intervals and added to a cuvette containing 500 μl phosphate buffer. The A_{410} was adjusted to zero against a reference cuvette containing 400 μl 50 mM CH_3NH_2 in phosphate buffer plus 500 μl phosphate buffer. After addition to each cuvette of 100 μl 10 mM DTNB ΔA_{410} was measured. Then each cuvette received 100 μl trypsin solution and after incubation for 120 s ΔA_{410} was again measured. Appearance of thiols with methylamine only (□); after further addition of excess trypsin (■). Active site titration of bound trypsin: 400 μl samples (mixture A) were added to cuvettes containing 390 μl phosphate buffer. The reference cuvette contained 390 μl phosphate buffer plus 400 μl 50 mM CH_3NH_2 in phosphate buffer. After addition of 100 μl trypsin, 100 μl STI and 10 μl *p*-NPGB to each cuvette the A_{405} was measured as in fig.1. Release of *p*-nitrophenol (○). α_2M was 2.66 μM in the cuvette. Determination of incorporation of CH_3NH_2: 250 μl α_2M solution was mixed with 250 μl 0.1 M $CH_3NH_2 \cdot HCl$ in phosphate (pH 8.0) containing 100 μCi $^{14}CH_3NH_2 \cdot HCl$. Aliquots (50 μl) of this mixture were removed and mixed with 50 μl 1 M NH_4HCO_3. Portions (80 μl) were spotted on 2 cm diam. pieces of Whatman 3MM paper and immediately dropped into cold 10% trichloroacetic acid and stirred. After extensive washing with 5% trichloroacetic and ethanol/acetone (1:1) the radioactivity was determined (●).

Furthermore, the incorporation of [^{14}C]methylamine into α_2M closely parallels the appearance of thiol groups and the decrease in the binding capacity of α_2M for trypsin, strongly indicating that the Glx-residue identified earlier as acceptor of methylamine [18] is part of a site in α_2M which mediates the covalent attachment of proteinase to α_2M. The maximal amount of [^{14}C]methylamine incorporated was found to be 4.0–4.2 mol/mol α_2M confirming the results in [18].

Fig.4 shows that the rapid appearance of thiol groups in α_2M can also be caused by denaturants such as guanidinium chloride or SDS, showing that the reactions which lead to the somewhat slower cleavage of the α_2M subunits into M_r 120 000 and M_r 60 000 fragments [15–17] depend on prior appearance of the thiol groups. Based on these observations we conclude that the methylamine-accepting Glx-residue [18] of the sequence –Pro–Tyr–Gly–Cys–Gly–Glu–Glx–Asn–Met–Val–Leu–Phe–Ala–Pro–Asn–Ile–Tyr–Val–Leu–Asp–Tyr–Leu– occurs in all 4 chains of α_2M as a reactive γ-glutamyl–thiol-ester. Preliminary

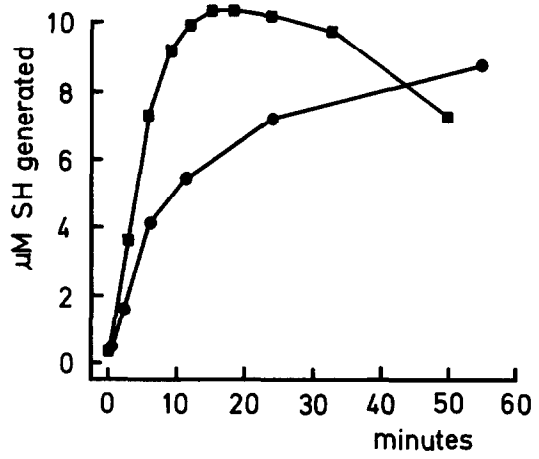

Fig.4. Appearance of thiol groups on denaturation of α_2M. α_2M solution (2.5 ml) was mixed with 1.25 ml 6 M guanidinium chloride buffer (pH 8.0) and kept at 50°C. Samples (300 μl) were removed at intervals and added to 600 μl phosphate buffer at room temperature. The reference cuvette contained 600 μl phosphate plus 300 μl 2 M guanidinium chloride in phosphate buffer (pH 8.0). After adjusting the A_{410} to zero, each cuvette received 100 μl 10 mM DTNB and ΔA_{410} was measured (■). In another experiment 1.0 ml α_2M solution was mixed with 200 μl 10% SDS in water and kept at 50°C. Samples (200 μl) were added to 700 μl phosphate buffer and the A_{410} adjusted to zero against a reference containing 700 μl phosphate plus 200 μl 1.67% SDS. After addition of 100 μl DTNB the ΔA_{410} was measured (●).

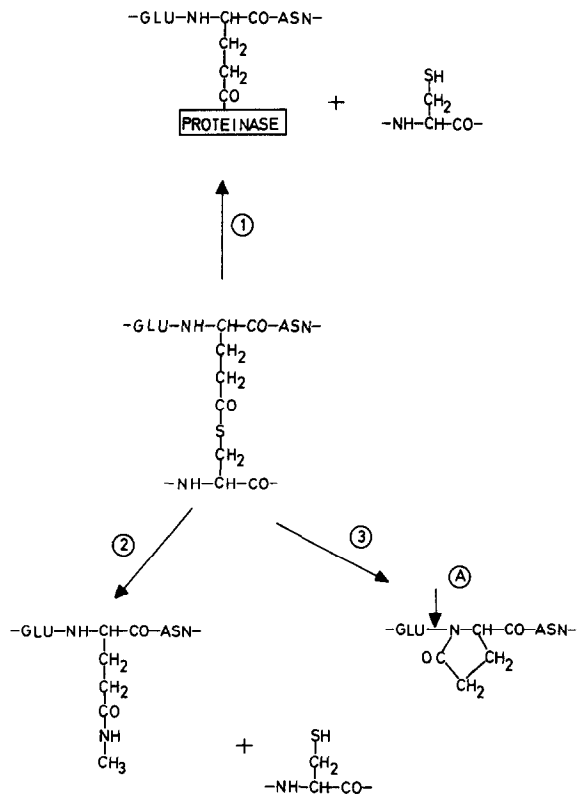

Fig.5. Schematic representation of the reaction paths of the proposed thiol-ester in the subunits of α_2M: (1) activation by proteinase; (2) reaction with methylamine; (3) effect of denaturation resulting in the formation of an internal pyroglutamyl residue in α_2M followed by secondary reactions. Cleavage (A) leads to formation of 120 000 M_r and 60 000 M_r fragments [15,16].

results show that the SH is contributed by a cysteine residue in the α_2M sequence (fig.5). As a result of cleavage by proteinases in the 'bait region' of α_2M this thiol-ester immediately becomes accessible to rapid reaction with a suitably oriented nucleophile, e.g., on the surface of the 'attacking' proteinase leading to an 'acyl transfer' reaction of the α_2M via its Glx-residue to the proteinase. Thus, the reactive site of α_2M resembles the thioester proposed as an intermediate in the transglutaminase catalyzed formation of isopeptide bonds [32]. The thiol-ester in α_2M is so labile that treatment with denaturants leads to its cleavage even at room temperature. Incubation of α_2M in buffers at pH <4.5 or pH >9.5 also makes the thiol groups appear rapidly.

The fact that in conditions of α_2M excess (sloping part of fig.1) the stoichiometry is 4:1 between thiol

released and trypsin added, for two chains cleaved, suggests that only half of the total thiol release is strictly correlated to the extent of proteolytic cleavage. This could mean that the structural change triggered by this proteolytic cleavage is 'transmitted' to the two uncleaved subunits in the α_2M tetramer, thus causing their two thiol-esters to be cleaved.

Note added

After this manuscript was typed we became aware of an abstract (Tack, B. F., Harrison, R. A., Janatova, J. and Prahl, J. W., 4th Int. Cong. Immunol. Paris, July 21–26, 1980, no. 15.1.19) indicating the presence of an internal thiol-ester in complement factor C3.

Acknowledgements

Outdated human plasma was obtained from The Blood Bank, Århus University Hospital through the courtesy of F. Kissmeyer-Nielsen, J. Jørgensen and S. Steenbjerg. This work was supported by The National Heart, Lung and Blood Institute, NIH (Bethesda, MD) grant no. HL16238.

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