Vol. 107, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1982 Pages 93-100

NASCENT α_2-MACROGLOBULIN-TRYPSIN COMPLEX: INCORPORATION OF AMINES AND WATER AT THE THIOL ESTERIFIED GLX-RESIDUES OF α_2-MACROGLOBULIN DURING ACTIVATION WITH TRYPSIN

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Received May 11, 1982

SUMMARY. During complex formation between α_2-macroglobulin and trypsin the internal thiol esters (one in each of the four M_r 180,000 subunits) are activated. In this activated state (nascent α_2-macroglobulin-trypsin complex) addition of low M_r amines lead to their covalent incorporation into α_2M. Evidence is presented showing that covalent binding of added amines occurs at the γ-carbonyl group of the Glx-residue in the thiol ester sequence:

-Met-Pro-Tyr-Gly-Cys-Gly-Glu-Glx-Asn-Met-
S-C=O

The tetrameric plasmaglycoprotein α_2-macroglobulin (α_2M), M_r 725,000, forms complexes with proteinases from all four classes, E.C.3.4.21-24. Recently, a model describing the interaction of α_2M with trypsin was presented (1,2). A key feature of this model is the hypothesis that the internal β-cysteinyl-γ-glutamyl thiol ester in each subunit of α_2M (3,4) can be activated by specific limited proteolysis in the bait region (5,6). In this state (nascent α_2M-trypsin complex) (6,7) the thiol esters are presumed to be highly reactive and can be cleaved rapidly by nucleophiles to form Cys-SH residues and Glx-residues substituted at the γ-carbonyl group (1-4,6,7).

This mechanism readily explains the formation of apparently covalent α_2M-proteinase complexes (5,8-12) and the simultaneous covalent binding of nucleophiles that happen to be present during activation of α_2M by proteinase

ABBREVIATIONS: α_2M, α_2-macroglobulin (human); dns, dansyl, 5-dimethylamino naphthalene sulfonyl; dns-cadaverine, 5-dns-(1,5)-diaminopentan; fluorescamine, 4-phenylspiro[furan-2-(3H),1'-phthalan]-3,3'-dion; PTH, phenylthiohydantoin; HPLC, high performance liquid chromatography; Hse, homoserine; Hsl, homoserine lactone; Glu (dns-cadaverine), glutamic acid-1-(5-dns-(1,5)-diaminopentyl)amide; Glu(putrescine), glutamic acid-4-(1,4-diaminobutyl)amide; PTC, phenylthiocarbamyl; T, trypsin.

0006-291X/82/130093-08$01.00/0

(6,7,11). Furthermore, the nascent forms of complement components C3b and C4b which both contain an activatable internal β-cysteinyl-γ-glutamyl thiol ester in their α'-chains (13-15) can also form apparently covalent bonds to a variety of compounds including cell-membranes, polysaccharides, immunoglobulins and low M_r nucleophiles (16-19). Here we show that the covalent incorporation of amines and water into α_2M during trypsin activation occurs at the γ-carbonyl group of the thiol esterified Glx-residues.

MATERIALS AND METHODS. Human α_2M was prepared by Zn^{2+}-affinity chromatography (1). Bovine trypsin (60% active) was from Boehringer, Mannheim, FRG. Dns-cadaverine, putrescine·2 HCl and fluorescamine were from Sigma, St.Louis, MO. 1-[^{14}C]-iodoacetic acid (54 Ci/mol) was from Amersham, UK. CNBr, iodoacetic acid and standard chemicals were from Merck, Darmstadt, FRG. Sephacryl S-200, Sephadex G-25F and G-50F were from Pharmacia, Uppsala, Sweden. Peptides were isolated by two-dimensional fingerprinting (20) using electrophoresis at pH 6.5 in the first dimension and paper chromatography in the second dimension (butanol:acetic acid:water:pyridine, 15:3:12:10 v/v (BAWP)). Peptides were localized by reaction with fluorescamine (0.001% in acetone) and by autoradiography. Peptides were eluted with 10% acetic acid and 20-80% hydrolysed with 6 M HCl at 110 °C for 20 h. The hydrolysates were analysed on a Beckman 121MB instrument. Automated sequence determination was performed in a Beckman 890C Sequencer (3). PTH-amino acids were determined by HPLC (21) using a Hewlett-Packard 1084B instrument.

EXPERIMENTAL. Complexes between α_2M and trypsin (α_2M:trypsin = 1:2 mol/mol) were formed by mixing 322 mg α_2M in 26 ml 0.1M Na-phosphate pH 8.0 containing 2 mM dns-cadaverine or 50 mM putrescine with 30 mg trypsin dissolved in 3 ml 1 mM HCl. After incubation for 5 min at room temperature thiol groups were alkylated with approx. 50 μCi ^{14}C-iodoacetic acid for 30 min followed by 60 mg unlabelled iodoacetic acid for 10 min. After dialysis against running tap water for 20 h the preparations were freezedried, redissolved in 30 ml 70% HCOOH and treated with 600 mg CNBr for 20 h at room temperature. After addition of 15 volumes water and freezedrying the degraded α_2M-trypsin complexes were redissolved in 10 ml 8 M urea, 0.2 M CH_3COONH_4, pH 5.0 and subjected to gel chromatography on Sephacryl S-200 (22) (Fig.1A,B).

RESULTS. The thiol ester in each M_r 180,000 subunit of α_2M occurs in the sequence: -Met-Pro-Tyr-Gly-Cys-Gly-Glu-Glx-Asn-Met- (3,4) and the β-cysteinyl groups appearing as a result of thiol ester cleavage (4 mol/mol α_2M at α_2M:trypsin = 1:2 mol/mol (1)) can be specifically labelled with ^{14}C-iodoacetic acid. Therefore, it can be expected that if covalent complex formation with trypsin and simultaneous nucleophilic substitution with added low M_r amines engage the thiol esterified Glx-residues, then the nine-residue fragment formed by CNBr degradation of ^{14}C-carboxymethylated α_2M-trypsin complex will co-

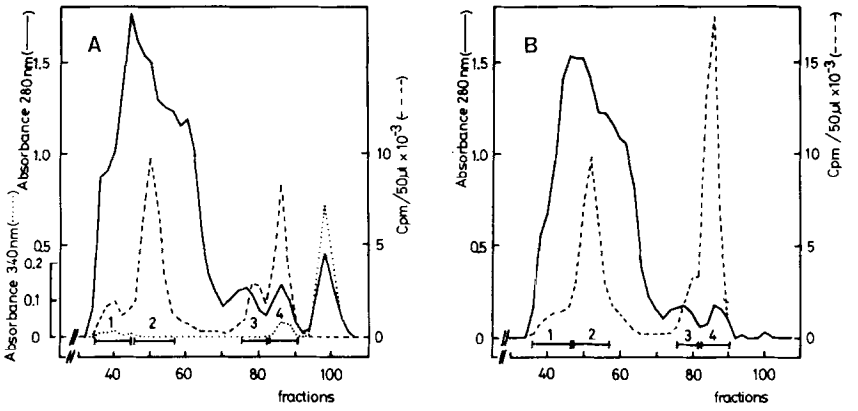

Figure 1. Separation of CNBr fragments from α_2M-trypsin complex formed in the presence of 2 mM dns-cadaverine (1A) or 50 mM putrescine (1B) on a column of Sephacryl S-200 (2.5 × 90 cm) equilibrated and eluted with 8 M urea, 0.2 M CH_3COONH_4 pH 5.0 at a flow rate of 20 ml/h. 5 ml fractions were collected. The separations were monitored by measuring A_{280} (——), A_{340} ((....), 1A) and by determining the radioactivity in 50 µl aliquots from every second fraction (----). Four pools (1-4) were collected as indicated. Pool 4 (1A) (fluorescent) was freezedried after desalting in 0.1 M NH_4HCO_3 on a column of Sephadex G-50F. The large peak eluting at fractions 94-106 (1A) was fluorescent and contained dns-cadaverine not removed by dialysis. Pool 4 (1B) and pools 2 and 3 from both experiments were separately desalted on a column of Sephadex G-25F in 0.1 M NH_4HCO_3 and freezedried. Distribution of ^{14}C label among peaks 1-4 (Fig.1A): 6, 50, 10, 25%, respectively, and (Fig.1B): 7, 33, 8, 43%, respectively.

elute during gel chromatography on Sephacryl S-200 either with the M_r 24,000 disulfide bridged fragment set from trypsin or with the low M_r fragments from α_2M

Gel chromatography of CNBr degraded α_2M-trypsin complex formed in the presence of 2 mM dns-cadaverine or 50 mM putrescine resulted in the separations shown in Fig.1A,B. Evidently the patterns are quite similar with two prominent peaks of radioactivity (peaks 2 and 4) eluting in the expected positions (22). The material in peak 1 (containing a small amount of high M_r ^{14}C-labelled fragments) was not investigated. From the corresponding pool 2 in large scale experiments we have isolated a number of peptides from trypsin that are covalently bound to the thiol esterified Glx-residues through ε-lysyl-γ-glutamyl cross-links (H.F.Hansen and L.Sottrup-Jensen, in preparation).

The material in peak 4 was further separated by fingerprinting (Fig. 2A,3). In addition to the weakly radioactive spots A,B,C,D (Fig.2A) and A,

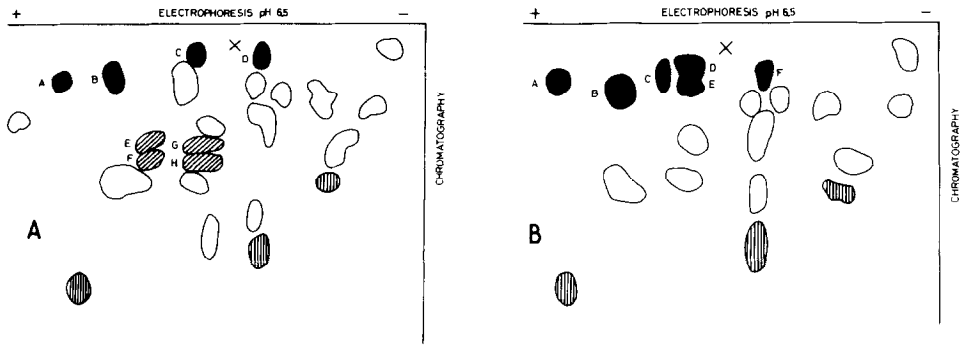

Figure 2. Fingerprint analysis of 50% of the pool 4 material from α_2M-trypsin complex formed in the presence of 2 mM dns-cadaverine (2A) and 50 mM putrescine (2B), respectively. The samples, dissolved in 10% CH_3COOH, were applied at (X) and were subjected to electrophoresis at pH 6.5 followed by chromatography in the perpendicular direction. The positions of the components of the marker mixture used are shown with vertical hatching (from left to right dns-SO_3H, dns-Arg and dns-Arg-Arg, respectively). Fluorescamine negative but radioactive peptides were localized by autoradiography and are shown in black (spots A,B,C,D,(Fig.2A) and spots A,B,C,D,E,F(Fig. 2B), respectively. Radioactive spots that in addition were fluorescent are shown crosshatched (spots E,F,G,H(Fig.2A)).

B,C,F (Fig.2B) two sets of strongly radioactive spots were found (E,F,G,H) (Fig.2A) and (D,E)(Fig.2B). Furthermore, spots E,F,G,H (Fig.2A) were strongly fluorescent. From the results of amino acid analysis it was evident that spots A,B,E,F,G,H (Fig.2A) and spots A,B,D,E (Fig.2B) all contained the expected nine-residue CNBr fragment from α_2M. The peptides in spots C,D (Fig. 2A) and C,F (Fig.2B) were obtained in very low yield (<1 nmol) and were not analysed further. Table 1 summarizes the yield, mobility and charge at pH 6.5 and the results of sequence determination of the peptides isolated from pool 4. The material in spots A,B (Fig.2A,B) contained the Hse/Hsl variants of the thiol ester fragment in which the Glx-residue has reacted with water to form a Glu-residue. The material in spots E,F and G,H (Fig.2A) was found to be the Hse/Hsl variants of the fragment in which the Glx-residue has reacted with dns-cadaverine to form a Glu-(dns-cadaverine)-residue. Upon sequence determination of the combined material from G and H (Fig.2A) a fluorescent PTH-derivative was found in step 7. This derivative was identified as PTH-Glu-(dns-cadaverine) as in (22). Likewise, the material in D,E (Fig.2B) yielded a PTH-derivative in step 7 that was identified as PTH-Glu-(PTC-putrescine)(22). From an additional experiment in which ^{14}C-labelled putrescine

Table 1. Yield, electrophoretic mobility and charge at pH 6.5 and results of sequence determination of peptides in which the thiol esterified Glx-residues have reacted with nucleophiles (dns-cadaverine, putrescine and water, respectively).

Spot	yield (nmol)	mobility/ charge[a)]	sequence
(Fig.2A)			
A	3	0.72/-3	Pro - Tyr - Gly - CmCys - Gly - Glu - Glu - Asn - Hse[b)]
B	4	0.56/-2	(0.3)(0.3)(0.2) (-) (0.1)(0.05)(0.05) (-) (-)
(Fig.2A)			
E,F	10	0.45/-2	Pro - Tyr - Gly - CmCys - Gly - Glu - Glx[e)] Asn - Hse[c)]
G,H	25	0.30/-1	(11) (11) (10) (5) (7) (6) (-) (1) (-)
(Fig.2B)			
A	2	0.73/-3	no sequence determined
B	4	0.52/-2	
(Fig.2B)			
D,E	60	0.28/-1	Pro - Tyr - Gly - CmCys - Gly - Glu - Glx[f)] Asn - Hse[d)]
			(18) (18) (22) (4) (21) (15) (-) (4) (-)

a) Mobility was related to size and charge (23), b, c, d) 0.5, 18 and 30 nmol, respectively, was used for sequence determination, e) identified as Glu-(putrescine), f) identified as Glu-(dns-cadaverine), see text for details. Values in brackets denote yields of PTH-derivatives as determined by HPLC (nmol). Lack of quantitative results is shown by (-).

had been incorporated into α_2M-trypsin complex approx. 80% of the label was found in the corresponding peak 4 (data not shown). The single low M_r ^{14}C-labelled peptide found in this experiment yielded the same sequence and the same characteristic PTH-derivative in step 7 (radioactive) as the material from D,E (Fig.2B).

DISCUSSION. This work shows that dns-cadaverine or putrescine present during α_2M-trypsin complex formation become incorporated covalently at the thiol esterified Glx-residues in nascent α_2M to form substituted Glx-residues. The reaction with water, leading to formation of a Glu-residue in the thiol ester fragment, accounts for approx. 9-16% of the total yield of low M_r substituted peptide material. In the absence of amines only 15-20% of the ^{14}C-label is recovered in the corresponding peak 4 (at α_2M:trypsin = 1:2 mol/mol). The label is predominantly found in the peptide that has a Glu-

residue, corresponding to the material in spots A,B (Fig.2A,B)(H.F.Hansen and L.Sottrup-Jensen, in preparation).

When amines are present during complex formation the fraction of label found in peak 4 increases, while that found in peak 2 decreases (Fig.1A,B) as anticipated (2,6). The finding that approx. 43% of the ^{14}C-label was located in peak 4 at 50 mM putrescine (Fig.1B) indicates that approx. 1.6 mol putrescine was bound per mol α_2M in this experiment. This estimate is somewhat lower than the earlier estimate (2.5 mol putrescine/mol α_2M) using a different technique (6,7). At 2 mM dns-cadaverine only approx. 25% of the label was found in peak 4 (Fig.1A), indicating covalent binding of approx. 0.9 mol dns-cadaverine per mol α_2M.

The amount of ^{14}C-label found in peak 2 varied from 50% (2 mM dns-cadaverine) to 33% (50 mM putrescine). In the absence of amines (α_2M:T = 1:2 mol/mol) 55-60% of the ^{14}C-label was found in peak 2 as determined from a number of experiments using different conditions of carboxymethylation (H.F. Hansen and L.Sottrup-Jensen, in preparation). The decrease in the extent of covalent complex formation caused by the presence of 50 mM putrescine (approx.45%) is similar to that reported earlier for 25 mM CH_3NH_2 using a different technique (2).

Since thiol ester cleavage is complete at the present conditions the finding that 55-60% of the ^{14}C-label is associated with peak 2 shows that covalent binding of trypsin engages 2.2-2.4 of the 4 thiol esterified Glx-residues. This indicates that a considerable fraction of the trypsin molecules bound covalently to α_2M at α_2M:T = 1:2 mol/mol (approx. 1.3 mol/mol α_2M (2,7,10,11)) is bound via two thiol esterified Glx-residues.

It is thus apparent that the degree of reaction of nascent α_2M-trypsin complex with water is relatively low even in the absence of amines, further indicating that most of the potential for formation of covalent bonds between α_2M and trypsin is actually utilized (at α_2M:trypsin = 1:2 mol/mol). This seems to contrast with the reaction between nascent C3b and C4b and

nucleophiles, in which cases only a few per cent of the activated molecules become bound to cell-surfaces or to antibody aggregates (16-19).

Recently, we located a crosslinking site in α_2M capable of incorporating dns-cadaverine or putrescine into a particular Gln-residue by factor $XIII_a$ (22). From the results presented here, it is evident that no incorporation of dns-cadaverine of ^{14}C-putrescine takes place at this site during complex formation.

The small peak of radioactivity (peak 3, Fig.1A,B) contained several labelled peptides in low yield. The major peptide, possibly formed by digestion of trypsin by contaminating chymotrypsin in the preparation of trypsin used, was isolated in a yield of 5 nmol. Upon sequence determination it became evident, that this peptide contained an ε-lysyl-γ-glutamyl crosslink between the thiol esterified Glx-residue of α_2M and lysine-239 of trypsin (chymotrypsinogen numbering). This lysine residue appears to be one of the major sites for covalent bond formation between α_2M and trypsin (H.F.Hansen and L.Sottrup-Jensen, in preparation).

ACKNOWLEDGEMENTS. We thank Lene Kristensen and Bente J.Christensen for excellent technical assistance. This work was supported by The National Heart, Lurg and Blood Institute, NIH (Bethesda, MD) grant HL 16238.

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