SEQUENCE LOCATION OF A PUTATIVE TRANSGLUTAMINASE CROSSLINKING SITE IN HUMAN α_2-MACROGLOBULIN

Steen B. MORTENSEN, Lars SOTTRUP-JENSEN†, H. Frede HANSEN, Diane RIDER*, Torben E. PETERSEN and Staffan MAGNUSSON

Department of Molecular Biology, University of Aarhus, 8000 Århus C. Denmark

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1. Introduction

The tetrameric plasmaglycoprotein α_2-macroglobulin (α_2M) consisting of 4 identical M_r 180 000 subunits forms complexes with proteinases from all 4 classes (EC 3.4.21–24) [1]. The bound proteinase retains activity towards small substrates and inhibitors [2,3]. α_2M–proteinase complexes are rapidly cleared from the circulation [4] and are in contrast with uncomplexed α_2M readily taken up by various cells in culture, e.g., fibroblasts [5] and macrophages [6,7] by receptor-mediated endocytosis. This uptake apparently involves cellular transglutaminases [8,9].

In addition to fibrin and cold-insoluble globulin α_2M is a major substrate for plasma transglutaminase (factor $XIII_a$) as shown by incorporation of dns-cadaverine into these proteins, when plasma is clotted in the presence of dns-cadaverine [10,11].

Here we show that ~93% of the covalent incorporation of dns-cadaverine or putrescine into α_2M occurs at the second Gln-residue in the sequence:
–Leu–Gln–Gln–Tyr–Glu–Met–

Abbreviations: α_2M, α_2-macroglobulin; dns-cadaverine, 1-(5-dimethylamino naphtalene sulfonyl)-1,5-diamino pentane; putrescine, 1,4-diamino butane; DTT, dithiothreitol; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin; α_2M(dnsc), α_2M covalently labelled with dns-cadaverine; α_2M(putrescine), α_2M covalently labelled with putrescine; PTI, bovine pancreatic trypsin inhibitor; PTC, phenylthiocarbamyl

* Present address: University of Wisconsin, Department of Medicine, Madison, WI 53706, USA

† To whom correspondence should be addressed

while the adjacent Gln-residue carries the rest. These Gln-residues are located 12 and 13 residues, respectively, before the major elastase cleavage site [12] in the bait region of α_2M.

2. Materials and methods

dns-Cadaverine and putrescine–2 HCl were from Sigma (St Louis MO). [1,4-^{14}C]Putrescine (122 Ci/mol) was from Amersham. CNBr, DTT, ICH_2COOH and standard chemicals were from Merck (Darmstadt) or from Fluka (Buchs). Sephacryl S-200 Sephadex G-25 and G-50 fine were from Pharmacia (Uppsala). SDS–PAGE was performed according to [13]. Hydrolysates of peptides were prepared and analysed as in [14]. Sequenator analysis and HPCL analysis of PTH derivatives were performed as in [14,15]. dns-Cadaverine containing fractions were localized by illumination with UV-light (Mineralight UVSL-58, UV products, San Gabriel CA). Incorporation of dns-cadaverine and putrescine into α_2M was determined by measuring the absorbance at 340 nm [11,16] and by scintillation counting [17], respectively. Incorporation of these amines in CNBr-fragments of α_2M was determined by amino acid analysis.

3. Experimental, results and discussion

Outdated human plasma was made 3 mM in dns-cadaverine or 10 mM in putrescine containing ^{14}C-labelled putrescine. The plasma was recalcified (final $CaCl_2$ conc. 25 mM) and kept for 3 h at room temperature (dns-cadaverine) [11] or for 16 h at 4°C

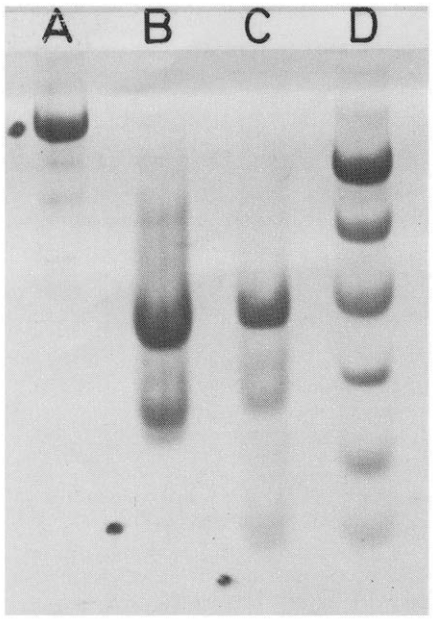

Fig.1. SDS–PAGE of α_2M(dnsc) (lane A) and of CNBr-degraded α_2M(dnsc) (lanes B,C). Samples were prepared in the presence (lanes A,C) or in the absence (lane B) of DTT. The positions of fluorescent bands as revealed by illumination with UV-light before staining with Coomassie brilliant blue are marked with India ink (black dots). The marker mixture (D) consisted of plasminogen (M_r 92 000), serum albumin (M_r 68 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 29 000), ribonuclease (M_r 14 000) and PTI (M_r 6500).

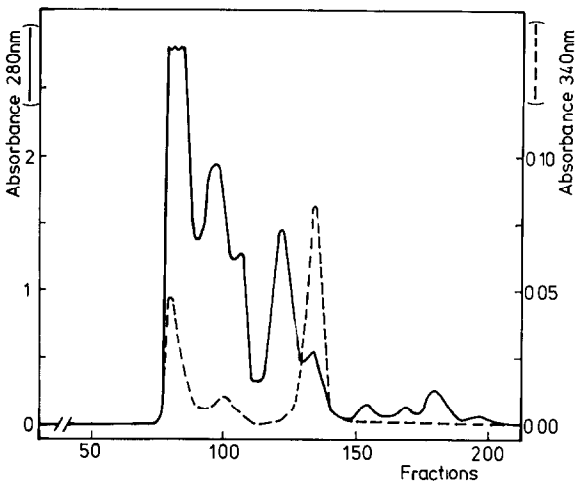

Fig.2. Gel chromatography of CNBr-fragments from 0.96 g α_2M(dnsc) on a column of Sephacryl S-200 (5 × 100 cm) equilibrated and eluted with 8 M urea, 0.2 M CH_3COONH_4 (pH 5.0). Flowrate was 40 ml/h and 10 ml fractions were collected. The separation was monitored by measuring the absorbance at 280 nm and at 340 nm. The fluorescent material eluting in fractions 131–140 was pooled, desalted on a column of Sephadex G-25 (5 × 40 cm) in 1 M CH_3COOH and freeze-dried. In a parallel experiment the CNBr-fragments from 0.24 g α_2M (putrescine) were separated on a 2.5 × 90 cm column of Sephacryl S-200 in the same solvent. The ^{14}C-labelled material eluted in the same relative position as the fluorescent material from α_2M(dnsc).

(putrescine) after clotting had occurred. α_2M was isolated from the serum by Zn^{2+}-affinity chromatography [17]. Fig.1A shows the result of reducing SDS–PAGE of α_2M(dnsc). In addition to the major M_r 180 000 band (fluorescent) minor bands at M_r 120 000 and M_r 60 000 (heat-cleaved α_2M subunits [18]) and M_r 85 000 (proteolytically cleaved subunits [19]) were also observed. For two preparations of α_2M-(dnsc) 1.55 and 2.06 mol dns-cadaverine were bound/mol α_2M as compared with 1.92 mol/mol in [11]. For one preparation of α_2M (putrescine) ~4.3 mol putrescine was bound/mol α_2M.

CNBr-degradation of α_2M(dnsc) revealed that the fluorescence originally found in the M_r 180 000 subunits of α_2M was associated with a disulfide-bridged low M_r CNBr-fragment (fig.1, lanes B,C). Fig.2 shows the result of gel chromatography of CNBr-degraded α_2M(dnsc) on Sephacryl S-200. Only the material eluting in fractions 126–140 showed yellowish-green fluorescence when illuminated by UV-light. Furthermore, all the ^{14}C-label of CNBr-degraded α_2M (putrescine) eluted in this position, showing that dns-cadaverine and putrescine was incorporated into the same fragment set. The pools from either experiment containing the bound amine were separately reduced with DTT and alkylated with ICH_2COOH and gel chromatographed on a column of Sephadex G-50 fine (2.3 × 72 cm) in 1 M CH_3COOH. Two pools (1,2) eluting at 0.48–0.56 and 0.80–0.88 bed volumes, respectively, were collected and freeze-dried. When redissolved in 0.1 M NH_4CHO_3 only the pool 2 material from α_2M(dnsc) was fluorescent and only the corresponding material from α_2M (putrescine) was radioactive.

Following amino acid analysis the material in pools 1 and 2 were identified as CB-K (59 residues) and CB-N (9 residues), respectively. These two CNBr-fragments are disulfide-bridged and form part of a completed 298 residue stretch containing the bait region [12] in α_2M (L. S., T. M. Stepanik, P. B. Lønblad, T. E. P., S. M., unpublished).

Table 1
Sequence of the dns-cadaverine or [^{14}C]putrescine labelled CNBr-fragment from α_2M

Position	Known sequence CB-N [20]	Residues detected (nmol) in CNBr-fragment obtained from			
		α_2M(dnsc)	fluorescence	α_2M (putrescine)	radioactivity (cpm)
1	Cys	CmCys (39)		CmCys (11)	16
2	Pro	Pro (46)		Pro (11)	19
3	Gln	Gln (37)		Gln (13)	15
4	Leu	Leu (50)		Leu (23)	15
5	Gln	Gln (32)	(+)	Gln (6)	16
6	Gln	Gln (30)	(+++++)	Gln (3)	59
7	Tyr	Tyr (36)	(++)	Tyr (7)	20
8	Glu	Glu (8)		Glu (2)	16
9	Hse	–		–	14

Fragment from α_2M(dnsc) (75 nmol) and 35 nmol of the fragment from α_2M (putrescine) was degraded. PTH amino acids were dissolved in 200 μl ethanol and 10 μl analysed by HPLC [15]. The solutions were illuminated by UV-light and the yellowish green fluorescence estimated qualitatively (+) (α_2M-(dnsc)). PTH (20 μl) from the ^{14}C-labelled CNBr-fragment was spotted on filterdiscs and the radioactivity determined by scintillation counting for 10 min [17]. For both sequenator runs an ~20% 'overlap' was created when passing the Pro-residue in step 2, due to incomplete cyclisation. Only 'in phase' yields are listed in the table. In steps where PTH-Gln was identified additional ~10% was recovered as PTH-Glu. In steps 5 and 6 additional peaks were observed in the HPLC chromatograms as discussed in the text

CB-N from α_2M(dnsc) contained 0.31 mol cadaverine/mol (6 M HCl, 150°C, 20 h) and CB-N from α_2M (putrescine) contained 0.92 mol putrescine/mol (6 M HCl, 110°C, 20 h). Table 1 shows the result of automated sequence determination of the two labelled fragments compared with the known sequence [20]. For both fragments it is evident that the major site of incorporation of dns-cadaverine and putrescine is Gln-6. As seen from the weak fluorescence observed in step 5 some incorporation of dns-cadaverine had also occurred at Gln-5. In addition to PTH-Gln a new peak was observed in steps 5 and 6 from both fragments in the HPLC chromatograms. The peak observed in CB-N from α_2M (putrescine) eluted ~0.9 min before PTH-Lys in the system of [15]. Following preparative isolation by HPLC and 'back' hydrolysis [21] glutamic acid and putrescine were found in about equimolar yields. The material in the peak from CB-N (α_2M dns-cadaverine) was fluorescent and eluted ~7 min later than PTH-Leu when a further gradient step from 35% ethanol [15] (Δ% ethanol/min = 0.9) was applied. By 'back' hydrolysis glutamic acid, cadaverine and dns-sulfonic acid were found. Thus, these two peaks most likely contained PTH-Gln (PTC-putrescine) and PTH-

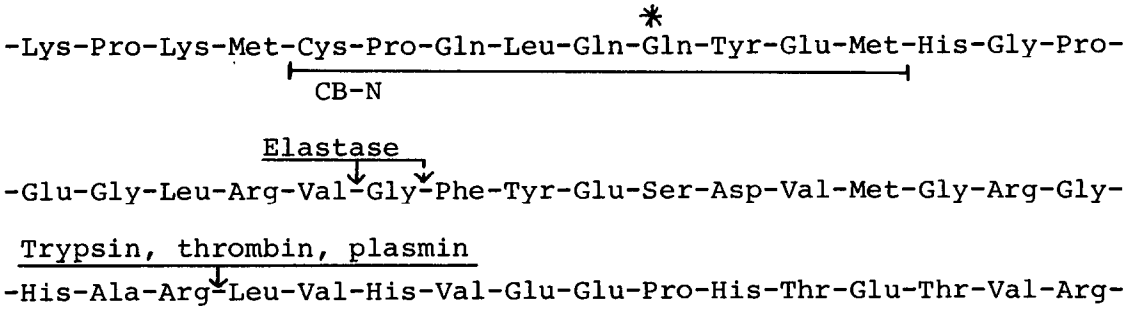

Fig.3. Amino acid sequence around the putative crosslinking site in α_2M, preceding the bait region. The position of CB-N is indicated by the bar. The cleavage sites in the bait region for elastase and trypsin (thrombin, plasmin) respectively, are shown by arrows. The sequence is deduced from the data in [12,20]. The Gln-residue that is the major site of incorporation of dns-cadaverine or [^{14}C]putrescine in α_2M is shown by (*).

Gln (dns-cadaverine), respectively. The yield of these derivatives in step 5 was ~7% of the yield in step 6. Assuming a repetitive yield of 90% and taking the ~20% 'overlap' generated at Pro-2 into account the yield of PTH-Gln for both sequenator runs in step 6 was ~2-times higher than that expected from the known content of dns-cadaverine and putrescine, respectively, in the fragments. However, the data clearly show that the incorporation of the amines into α_2M occurs at Gln-6 (~93%) and Gln-5 (~7%) of CB-N.

As shown in fig.3 the major site of incorporation is located 12 residues from the major elastase cleavage site [12] in the bait region of α_2M utilising the data in [12,20].

These results show that not only dns-cadaverine [10,11] but also putrescine become covalently incorporated into α_2M when present during clotting of plasma. Recently, it was reported that α_2M(dnsc) had essentially the same proteinase binding capacity and methylamine reactivity as native α_2M [11] indicating that the thiol esterified Glx-residues of α_2M [17] were not the site of incorporation of dns-cadaverine. Since no fluorescence or radioactivity was found in the elution position of the CNBr-fragment Pro–Tyr–Gly–Cys–Gly–Glu–Glx–Asn–Hse [14], containing the cleaved thiolester (eluting at fractions 160–175, fig.2) (L. S., unpublished) it is evident that no reaction with these amines has taken place with the reactive thiol esterified Glx-residues under the present conditions.

The functional significance of the putative cross-linking site thus localized in α_2M is at present unclear. It appears that cellular transglutaminases are involved in receptor-mediated endocytosis of α_2M–proteinase complexes [8], possibly at a stage that regulates the recycling of the receptors [9]. The demonstration that α_2M(dnsc) or α_2M(dnsc)–trypsin complexes could not compete with α_2M–trypsin for uptake into fibroblast-cultures [11] show that prior reaction of α_2M with dns-cadaverine (and presumably also with putrescine) at the Gln-residues localised here interferes with an important step in the uptake mechanism for α_2M–proteinase complexes. Whether the site identified here is utilised for the cellular tranglutaminases is not yet known.

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