Domain structure of human α_2-macroglobulin

Characterization of a receptor-binding domain obtained by digestion with papain

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Digestion of methylamine-treated α_2-macroglobulin (α_2M·MA) with catalytic amounts of papain at pH 4.5 has been investigated. Cleavage of Lys(1313)-Glu resulted in two major products, which could be separated by gel chromatograhy: a large disulfide bridged fragment set nearly the size of intact α_2M·MA, and an 18 kDa fragment, constituting the carboxy-terminal domain of α_2M. This domain contained the receptor recognition site, exposed as a result of cleavage of the internal β-cysteinyl-γ-glutamyl thiol esters in α_2M. Compared with α_2M-trypsin complex the apparent affinity for binding to rat hepatocyte receptors was 0.1 and 2% at 4 and 37 C, respectively. The receptor-binding domain presumably forms a compact globular β-barrel-type structure, stable at pH 2.5–9.0. Chemical modification experiments suggest that receptor binding is contributed by a determinant formed by the precise folding of the polypeptide chain.

α_2-Macroglobulin Domain structure Proteolysis Papain Cellular receptor Chemical modification

1. INTRODUCTION

α_2M-proteinase complex formation is initiated by specific limited proteolysis in the activation cleavage region (residues 681–686 [1]). This triggers a conformational change, resulting in the generation of tight-fitting binding sites for proteinases, one in each dimeric unit [2]. In addition, previously concealed recognition sites appear in α_2M, important for the rapid clearance of α_2M-proteinase complexes by receptor-mediated endocytosis [3–8]. Methylamine-inactivated α_2M is also rapidly cleared [3,7,8]. Using digestion with an extracellular proteinase from *Lysobacter enzymogenes* at pH 4.5 the receptor recognition site was recently localized to a domain containing the carboxy-terminal 138 residues [9], which also contains epitopes of neoantigens appearing after thiol ester cleavage [4,10]. Here we report, in the course of probing the domain structure of α_2M by proteolytic digestion, that the same domain can be obtained in high yield by digestion with the readily available papain at pH 4.5. In addition, the effect on receptor binding by chemically modified versions of the receptor-binding domain is reported.

Abbreviations: α_2M, α_2-macroglobulin (human); α_2M·MA, α_2-macroglobulin treated with CH_3NH_2 and ICH_2CONH_2; EDC, ethyldimethylaminopropyl carbodiimide; DTT, dithiothreitol; GuHCl; guanidinium chloride; α_2MT, α_2-macroglobulin-trypsin complex.

2. MATERIALS AND METHODS

Human α_2M prepared as in [11] was treated with CH_3NH_2 and ICH_2CONH_2 [12]. Crystalline papain was from Boehringer, Mannheim. SDS-PAGE was performed according to [13] using

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10–20% gradient gels. Procedures for amino acid analysis and sequence determination were reported in [14]. Determination of binding of α_2M-trypsin complex to rat hepatocytes at 4°C, and uptake at 37°C were done as in [15,16]. Lys residues in the receptor-binding domain were modified by succinylation and citraconylation at pH 9.5 [17], Arg residues by reaction with 1,2-cyclohexanedione at pH 8.0 [18], and -COOH groups by reaction with EDC at pH 6.1 [19]. All modified derivatives were soluble to at least 2 mg/ml at pH 7–8.

Digestion of α_2M · MA (770 mg dissolved in 100 ml of 0.1 M CH_3COONa, pH 4.5) with papain (12 mg dissolved in 6 ml of 0.1 M CH_3COONa, 5 mM cysteine, pH 5.0) was carried out for 20 h at room temperature (S:E = 1:0.3 mol/mol, equal to 64:1 w/w). At the end of digestion Tris base was added and papain inactivated by addition of 1 ml of 0.5 M IHC_2CONH_2 at pH 8.0. The digest was analyzed by SDS-PAGE (fig.1, inset) and 80% of the material separated by gel chromatography (fig.1). The material in pool 1 (large fragment set) was dialyzed into 50 mM Tris-HCl, 0.1 M NaCl, pH 7.4, and concentrated by ultrafiltration (Amicon PM 10 membrane) to 34 mg/ml (assuming E (1%, 280 nm, 1 cm) = 9.0). The volume and the buffer concentration of the material in pool 2 (small fragment set) was reduced to one-fifth by ultrafiltration. A subsequent DEAE-Sephacel step (not shown) using gradient elution with NH_4HCO_3 yielded a nearly symmetrical peak eluting between 0.15 and 0.25 M NH_4HCO_3. This material was dialyzed into the same Tris buffer and concentrated to 4 mg/ml by ultrafiltration. Based on a content of 7 Phe residues and 4 Arg residues, and a molecular mass of 18 000 (see below) E (1%, 280 nm, 1 cm) was estimated at 5.5. The preparative yield was 50 mg (approx. 80%).

3. RESULTS

Although it has been recognized that α_2M · MA is very resistant towards proteinases [20] extensive fragmentation can be obtained using a 2–4-fold molar excess of trypsin, chymotrypsin, elastase, and papain (not shown). Using small amounts of papain a relatively limited digestion took place at pH 4.5 (fig.1). The material of low molecular mass separated by gel chromatography (pool 2, fig.1) consisted of two major fragments (20 and 17 kDa, reducing conditions), while the remainder of α_2M · MA (pool 1, fig.1) was a large disulfide bound set of several fragments. At pH 4.5 and room temperature the small fragment set was nearly completely released from α_2M · MA in 4–6 h at papain:α_2M · MA ratios ranging from 1:20 to 1:80 (w/w). At pH 7.0 these fragments were only seen by using larger amounts of papain, which also resulted in a different and more extensive fragmentation of α_2M · MA (not shown). The following sequence was obtained from the small fragment set in a purity of more than 95%: Glu-Glu-Phe-Pro-Phe-Ala-Leu-Gly-Val-Gln-Thr-Leu-Pro-, showing

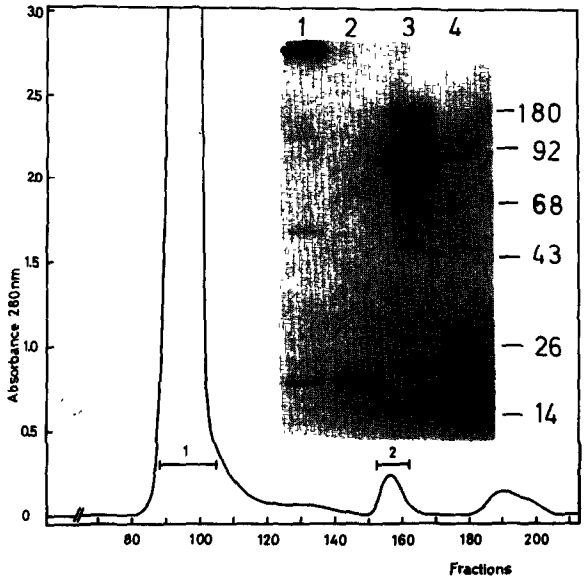

Fig.1. Gel chromatography of papain-digested α_2M · MA. A column of Sephacryl S-200 (5 × 107 cm) was equilibrated and eluted with 0.1 M NH_4HCO_3, pH 8.2, at a flow rate of 100 ml/h. 10-ml fractions were collected and two pools (1 and 2) were made. In a parallel experiment where papain was not inactivated by reaction with ICH_2CONH_2 activity measurements using N-benzoyl-L-arginine-p-nitroanilide showed that more than 95% of the papain used for digestion was associated with pool 1. Inset: SDS-PAGE analysis of papain-digested α_2M · MA and purified 18 kDa fragment. Lanes: 1 and 3, whole digest; 2 and 4, fragment set from pool 2; 3 and 4, DTT reduced samples. The position and size (kDa) of electrophoresis markers are indicated (reduced α_2M-subunit, plasminogen, albumin, ovalbumin, chymotrypsinogen, ribonuclease).

that the major fragments arose by specific cleavage at Lys(1313)-Glu in α_2M [1]. Presumably they extend to the carboxy-terminus of α_2M as judged from agreement with the amino acid composition for the stretch 1314–1451 in α_2M: Asx 13.2 (14); Thr 7.4 (9); Ser 13.6 (15); Glx 13.7 (13); Pro 8.4 (8); Gly 3.9 (4); Ala 9.5 (9); Val 12.6 (15); Cys 1.4 (2); Met 2.3 (3); Ile 5.1 (6); Leu 12.7 (12); Tyr 5.9 (6); Phe 7.4 (7); Lys 8.6 (8); His 2.6 (3); Arg 4.1 (4). The molecular mass of this stretch is 15331. Including carbohydrate bound to Asn-1401 [1] the molecular mass is estimated at 18 kDa. The apparent size of 20 kDa estimated from reducing SDS-PAGE is consistent with the anomalous behaviour of carbohydrate containing polypeptides in this analysis. The fragments of apparent size 17 kDa and less probably represent minor variants, in which part of the carbohydrate bound to Asn-1401 is missing [1,9].

The large and the small fragment sets were tested for their ability to compete with ^{125}I-α_2M-trypsin complex for binding to rat hepatocytes at 4°C. As seen from fig.2 the small fragment set was able to completely displace α_2M-trypsin from its hepatocyte receptors. The affinity of this fragment for binding to the α_2M receptors was approx. 0.1% that of binding of α_2M-trypsin (apparent $K_d \sim 0.3$ μM vs 0.2 nM [15]). This figure is similar to that obtained for binding of the small fragment set to α_2M receptors in fibroblasts [9]. In uptake studies at 37°C (not shown) the potency was about 2% (apparent $K_m \sim 0.5$ μM vs 10 nM).

Incubation at pH 9.0, 3.5, and 2.5 for 20 h (room temperature) had no effect on the ability of the small fragment set to compete with ^{125}I-α_2M-trypsin for binding. Incubation with pepsin (E:S = 1:100 mol/mol, pH 2.5) for 1 h at room temperature had no effect on receptor binding, and sequence analysis showed that the fragment was still intact. After incubation in 6 M GuHCl for 20 h (fig.2) or at pH 12.0 for 15 min approx. 10% of the receptor-binding activity was still present. In contrast, the affinity of the small fragment set was reduced to less than 1% in samples that had been exposed to 50% HCOOH for 20 h, or had been reduced with DTE in the absence or presence of GuHCl. Succinylation of Lys residues and modification of -COOH groups by EDC also reduced receptor binding to less than 1%, while modification of Arg residues by 1,2-cyclohexanedione had

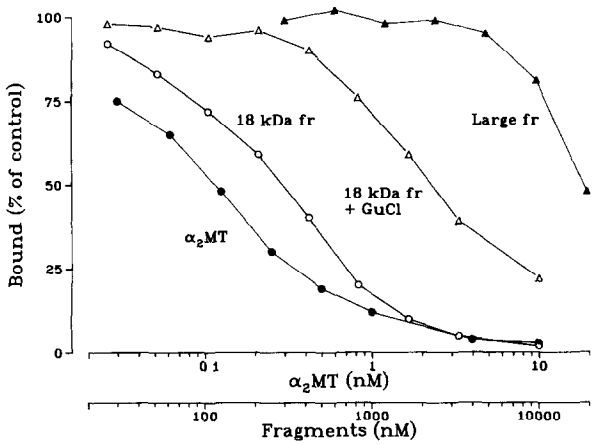

Fig.2. Binding of ^{125}I-α_2M-trypsin complex to rat hepatocytes in the presence of varying amounts of unlabelled α_2M-trypsin, and fragments from pools 1 and 2 (fig.1). Hepatocytes (4×10^5 in 250 μl) were incubated with 30 pM labelled complex (about 5000 cpm/tube) and unlabelled ligand as indicated for 20 h. The curves represent the mean values of 4 independent experiments. The amount of tracer bound to the cells in the absence of unlabelled ligand varied from 16 to 25% in individual experiments, and binding is therefore expressed in percent of the control value. The small (18 kDa) fragment set was, relative to α_2M-trypsin, more effective in competing with the tracer at high than at low concentration. Its potency was calculated as 0.4×10^{-3}, i.e. 0.16% per α_2M-trypsin subunit. This should be taken as an approximation since the competition curves were not parallel.

no effect. No receptor binding could be demonstrated in a tryptic digest of citraconylated receptor-binding fragment containing the following peptides from α_2M in which the Lys residues had been deblocked at pH 1.5 for 6 h: 1314–1350, 1351–1378, 1379–1384, 1385–1417, and 1418–1451.

In all experiments with modified versions of the small fragment set a concentration of 5–10 μM was used. At this concentration essentially no binding of ^{125}I-α_2M-trypsin occurs in the presence of the native fragment set (fig.2).

4. DISCUSSION

The carboxy-terminal 138 residue fragment evidently constitutes a relatively stable domain, which can readily be separated from the remainder of α_2M by cleavage at Lys(1313)-Glu at pH 4.5 as also observed recently [9]. Since this domain competes

effectively with α_2M-trypsin complex for binding to hepatocytes, it presumably forms a discrete domain in α_2M containing all structural information required for receptor recognition. However, the apparent affinity for receptor binding/uptake was only about 0.1–2% that of α_2M-trypsin complex, indicating that the receptor recognition domains (two in each dimeric proteinase binding unit [4]) may be uniquely positioned in intact α_2M-proteinase complex for recognition by the cellular receptor.

The receptor-binding domain of α_2M is largely constituted by alternating β-strands and reverse turns, presumably forming a β-barrel type of domain [21]. This domain is stable in the pH range 2.5–9.0 and is relatively tight as probed by its resistance to proteolytic digestion. However, reduction of its internal disulfide bridge or exposure to 50% HCOOH leads to complete loss of receptor binding, indicating that the cellular receptor recognizes a determinant formed by the precise folding of the polypeptide chain in the domain. This is further substantiated by the lack of receptor binding of a mixture of relatively large tryptic peptides containing internal Lys residues covering the stretch 1314–1451 in α_2M. Since modification of Lys, and Glu and Asp residues results in loss of binding affinity, it is likely that the receptor-binding determinant is part of one or more external loops, presumably also containing hydrophobic residues. At present these cannot be unambiguously pointed out [21].

The site of cleavage (Lys(1313)-Glu) is located in a short hydrophilic stretch [1] connecting two domains of α_2M (fig.3) and is exposed at pH 4.5. Although the exposure of the receptor recognition site in α_2M apparently is dependent on a conformational change effected by thiol ester cleavage the mechanism is not known. For transmitting conformational changes in the domain(s) controlling the state of the thiol ester to the carboxy-terminal receptor-binding domain the disulfide bridge Cys(898)-Cys(1298) [1] (fig.3) could be important by juxtaposing these domains in the native structure of α_2M. In view of the common ancestry of α_2M, C3 and C4 [22,23] it is possible that the domains in C3 and C4 corresponding to the receptor recognition domain in α_2M (fig.3) are important for recognition by one or more of the cellular receptors known to interact with activated C3 and C4 or their degradation products. Curiously, C3 and C4 also contain a similarly located disulfide bridge originating from a position close to the thiol ester domain and spanning across several hundred residues in sequence [24,25].

The finding here that more than 95% of the papain used for digestion of α_2M · MA was associated with the large fragment set (fig.1, legend) confirms earlier reports showing that α_2M · MA slowly binds proteinases [20], and indicates that the small carboxy-terminal domain is not involved in forming the structures in α_2M necessary for tight proteinase complex formation.

The demonstration that receptor binding of α_2M is confined to a discrete domain prompts further studies on the domain structure of α_2M, particularly with regard to a more detailed understanding of its structural and functional relationship with the complement proteins C3 and C4.

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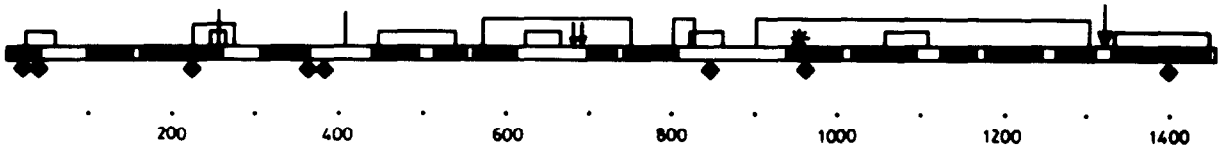

Fig.3. Schematic representation of the structure of the α_2M subunit, emphasizing common domains in α_2M, C3 and C4 (black segments) [22,23]. The disulfide bridge pattern is indicated, and the dimeric proteinase binding unit of α_2M contains two interchain disulfide bridges (L.S., unpublished). Carbohydrate groups are shown by filled diamonds, and the thiol ester site indicated by an asterisk. The sites of activation cleavage in the bait region are shown with small arrows. The site of cleavage by papain leading to separation of the carboxy-terminal receptor-binding domain from the large disulfide bridged 'core' is shown by a large arrow.

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