The structure of α_2 -macroglobulin–methylamine after papain digestion as determined by electron microscopy

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α_2 -Macroglobulin–methylamine (α_2 M–CH₃NH₂) was digested with papain at pH 5.0. The major 600 kDa fragment was purified by molecular-exclusion chromatography. In a non-denaturing gel-electrophoresis system, the 600 kDa fragment migrated in a single band at a rate that was comparable with that for the untreated α_2 M–CH₃NH₂. The elution volume of the 600 kDa fragment on Superose-6 was slightly increased. In primary cultures of rat hepatocytes, cellular uptake of ¹²⁵I- α_2 M–CH₃NH₂ was not affected by the 600 kDa fragment, confirming the results of other investigators. The 600 kDa fragment was negatively stained with uranyl formate and analysed by transmission electron microscopy. The major structural characteristics of the parent protein (α_2 M–CH₃NH₂) remained intact. The most common image included prominent lateral walls and two centrally located regions of stain exclusion termed 'paddle structures'. The distance between the paddle structures was equivalent in α_2 M–CH₃NH₂ and the 600 kDa fragment [$\approx$ 13.5 nm (135 Å)]. By contrast, the lateral walls in the 600 kDa fragment were decreased in length by $\approx$ 0.37 nm (37 Å) (19%). It is proposed that the 600 kDa structure retains the 'hollow cylinder' shape of α_2 M–CH₃NH₂. The structure of the cylinder is formed by the lateral walls and four paddle structures (only two are imaged, owing to overlapping). The paddle structures in the 600 kDa fragment are intact and relatively closer to the apices of the molecule, owing to the decrease in lateral wall length. Since the α_2 M receptor-binding sites are removed by papain digestion, the studies presented here support the location of the receptor-binding sites near the apices of the lateral walls.

INTRODUCTION

α_2 -Macroglobulin (α_2 M) is a large plasma glycoprotein (718 kDa) that inhibits proteinases of all four major classes [1]. The native protein consists of four identical subunits [2–4]. Intersubunit disulphide bonds bridge subunit pairs, forming covalent units with a molecular mass of approx. 360 kDa [2]. Association of subunit pairs in the intact whole molecule is due to strong non-covalent interactions [2,4].

Proteinases initiate a reaction with α_2 M by cleaving one of a series of susceptible peptide bonds in an area of the α_2 M subunit termed the 'bait region' [1,2,5]. This interaction causes a conformational change in the α_2 M molecule, trapping the enzyme so that a non-dissociable complex is formed, even though the enzyme active site is regenerated. α_2 M–proteinase complexes migrate more rapidly than the unchanged or native inhibitor in non-denaturing gel systems [5]. This finding has led to the terms 'slow form' and 'fast form' to designate the conformations of α_2 M before and after reaction with proteinase respectively. A similar conformational change may be induced in α_2 M by small primary amines such as methylamine (the resulting preparation will be referred to as ' α_2 M–CH₃NH₂') [5,6]. Receptors for α_2 M present on macrophages, hepatocytes and fibroblasts recognize only the transformed conformation or fast form of α_2 M (enzyme- or amine-treated) [7–10].

α_2 M conformational change has been studied extensively by electron microscopy. For the most part these investigations have demonstrated that the slow- and fast-form structures are highly distinct. Negatively stained images of native α_2 M have been likened to a 'padlock' structure [11], a 'doughnut' [12,13], a tetramer of spherules [13] and, most recently, a doughnut surrounded by four spherules [14]. Considerable variability is usually evident in these electron micrographs. By contrast, fast-

form α_2 M yields a readily reproducible and rather consistently oriented negatively stained image [11–14]. The structure basically resembles the letter 'H', although the central bar connecting the lateral arms may not be prominent [15]. A single small circular region of stain exclusion (termed the 'paddle structure' in the present paper) is usually observed on either side of the 'hypothetical' central bar [15,16]. α_2 M–proteinase and α_2 M–CH₃NH₂ are not significantly different in negatively stained preparations, although the proteinase may be identified centrally in the inhibitor structure in some cases [15,16].

At pH 4.5–5.0, α_2 M–CH₃NH₂ is cleaved by papain, causing the release of an 18 kDa fragment from the C-terminus of each subunit [17]. A proteinase isolated from the bacterium *Lysobacter enzymogenes* causes a similar change in the structure of α_2 M–CH₃NH₂ [18]. In both cases, cell-surface receptor-binding activity co-purifies with the small 18 kDa fragment; however, the affinity is significantly reduced [17–19]. The residual 600 kDa portion of α_2 M–CH₃NH₂ retains no affinity for the α_2 M receptor, as determined in experiments with cells in culture or *in vivo* in mice [17–19].

By using immuno-electron microscopy, the location of the α_2 M receptor-binding site has been identified near the four tips of the lateral walls in the transformed H structure [20]. If the acidic proteinase treatments truly cause dissociation of the receptor-binding domains in α_2 M–CH₃NH₂, then the 18 kDa fragments should represent the apices of the lateral walls, as suggested by the antibody studies. In the present investigation the structure of the 600 kDa fragment (α_2 M–CH₃NH₂ after papain digestion) has been studied by electron microscopy, f.p.l.c., native gel electrophoresis and receptor-binding experiments. Our studies indicate that the major features of the α_2 M–CH₃NH₂ structure remain intact after papain digestion, without evidence of denaturation. A significant decrease in the

Abbreviations used: α_2 M–CH₃NH₂, α_2 -macroglobulin–methylamine; EBSS, Earle's balanced salts solution; PBS, 20 mM-sodium phosphate/150 mM-NaCl, pH 7.4.

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length of the lateral walls was measured. By contrast, the structure of the two paddles and the distance between the paddles was not changed.

MATERIALS AND METHODS

Materials

Iodoacetamide and CH_3NH_2 were purchased from Sigma (St. Louis, MO, U.S.A.). Electrophoresis reagents were purchased from Bio-Rad (Richmond, CA, U.S.A.). Papain was purchased from Sigma. Human $\alpha_2\text{M}$ was purified from plasma by the method of Kurecki *et al.* [21] as modified by Imber & Pizzo [7]. $\alpha_2\text{M}$ was modified with CH_3NH_2 by dialysing the native inhibitor against 200 mM- CH_3NH_2 /50 mM-Tris/HCl, pH 8.2, for 6 h at 22 °C and then exhaustively against 20 mM-sodium phosphate/150 mM-NaCl, pH 7.4 (PBS) at 4 °C.

Digestion of $\alpha_2\text{M}-\text{CH}_3\text{NH}_2$ with papain

$\alpha_2\text{M}-\text{CH}_3\text{NH}_2$ (6.0 mg) was dialysed against 100 mM-sodium acetate and then allowed to react with papain (0.3 mg) in 50 mM-sodium acetate/1.0 mM-cysteine, pH 5.0. After incubation for 20 h at 22 °C, the pH of the solution was raised to 7.5 by the addition of Tris/HCl, pH 8.0. Iodoacetamide (5.0 mM) was added to inactivate the papain. After 30 min, the reaction mixture was fractionated on Ultrogel AcA-22 (1.0 cm $\times$ 50 cm) in PBS.

Papain-treated $\alpha_2\text{M}-\text{CH}_3\text{NH}_2$ was analysed by size-exclusion chromatography on a pre-packed Superose-6 column (Pharmacia LKB Biotechnology, Piscataway, NJ, U.S.A.) using a Perkin-Elmer LC system equipped with a diode-array detector (LC-235) coupled to a Gibson fraction collector (FC 203). The chromatography buffer was PBS and the flow rate was 0.4 ml/min. The void volume (V_0) of the column was 6.40 ml as estimated with Blue Dextran (2000 kDa). The total accessible volume, defined as the sum of V_0 and the pore volume, was 19.7 ml as determined with NaN_3 .

PAGE

Chromatography fractions were analysed by SDS/PAGE under reducing and non-reducing conditions using the imidazole/Hepes, pH 7.5, buffer system described by McLellan [22] and modified by Gonias *et al.* [23].

F.p.l.c.-purified native $\alpha_2\text{M}$, $\alpha_2\text{M}-\text{CH}_3\text{NH}_2$ and papain-treated $\alpha_2\text{M}-\text{CH}_3\text{NH}_2$ were subjected to native gel electrophoresis at pH 7.5 [22]. The gel was stained with 0.25% (w/v) Coomassie Brilliant Blue R-250 in methanol/acetic acid/water (4:1:5, by vol.).

Radioiodination

$\alpha_2\text{M}-\text{CH}_3\text{NH}_2$ was radioiodinated with Na^{125}I (Amersham) using Iodobeads as described by the manufacturer (Pierce Chemical Co.). The specific radioactivity was 2.0 $\mu\text{Ci}/\mu\text{g}$.

Binding studies

Primary cultures of hepatocytes were prepared by using Sprague-Dawley rats as previously described [24]. The cells were cultured in 35 mm-diameter collagen-coated plates (15 μg of collagen/well). Before starting an experiment, the wells were washed twice with Earle's balanced salts solution (EBSS) with 10 mM-Hepes, pH 7.4, and BSA (10 mg/ml). Association of $^{125}\text{I}-\alpha_2\text{M}-\text{CH}_3\text{NH}_2$ (1 nM) with hepatocytes was studied at 37 °C in the presence of different concentrations (0.2–200 nM) of unlabelled $\alpha_2\text{M}-\text{CH}_3\text{NH}_2$ or 600 kDa fragment. After 3 h, incubations were terminated by separating the medium from the cells. The cells were washed three times with EBSS/Hepes, pH 7.4 and lysed with 0.1 M-NaOH/2.5% (w/v) SDS. Cell-associated radioactivity was determined in a γ -radiation counter.

This procedure has been described in greater detail elsewhere [24].

Electron microscopy

Solutions of $\alpha_2\text{M}$ (50 $\mu\text{g}/\text{ml}$) were adsorbed on thin carbon films for 1 min. The films were then transferred to 2.0% glutaraldehyde for 10 min and washed extensively with deionized water. Films were stained with 2.0% uranyl formate for 60 s, transferred to 300-mesh nickel hexagon grids and air-dried at 28 °C.

Air-dried grids were analysed by conventional transmission electron microscopy using a Zeiss 902 electron microscope at 80 kV. All photomicrographs were obtained at a direct magnification of $\times 148\,000$ and $\times 240\,000$.

Image analysis

Photomicrograph negatives obtained at a direct magnification of $\times 148\,000$ were enlarged 2.5 times to form positive prints. Measurements were then made using the prints and a Laboratory Computer Systems Micro-Plan II Image Analyzer. The reported values represent the mean $\pm$ 1 s.d. ($n = 25$). Statistical significance was determined using the Student *t* test for paired data.

RESULTS

Chromatography of papain-digested $\alpha_2\text{M}-\text{CH}_3\text{NH}_2$

Fractionation of papain-treated $\alpha_2\text{M}-\text{CH}_3\text{NH}_2$ on Ultrogel AcA 22 yielded two major peaks. As determined by SDS/PAGE (results not shown), these peaks corresponded to the 600 kDa and 18 kDa fragments described by Sottrup-Jensen *et al.* [17]. The 600 kDa and 18 kDa fragments were analysed by f.p.l.c. using a Superose-6 column. The mean elution volume of the 600 kDa fragment (10.88 ± 0.12 ml, $n = 4$) was slightly increased compared with that of $\alpha_2\text{M}-\text{CH}_3\text{NH}_2$ before reaction with papain (10.51 ± 0.04 ml).

Native gel electrophoresis

$\alpha_2\text{M}-\text{CH}_3\text{NH}_2$, the 600 kDa fragment and native $\alpha_2\text{M}$ were subjected to native gel electrophoresis (Fig. 1). $\alpha_2\text{M}-\text{CH}_3\text{NH}_2$ demonstrated the well-characterized increase in mobility relative to native $\alpha_2\text{M}$. The 600 kDa fragment migrated as a single defined band. The mobility was comparable with that of $\alpha_2\text{M}-\text{CH}_3\text{NH}_2$.

Fig. 1. PAGE of the 600 kDa fragment

Electrophoresis was under non-denaturing conditions. Lane a, native $\alpha_2\text{M}$; lane b, $\alpha_2\text{M}-\text{CH}_3\text{NH}_2$; lane c, $\alpha_2\text{M}$ -trypsin; and lane d, 600 kDa fragment.

Fig. 2. Electron micrographs of α_2 M-CH₃NH₂ and the 600 kDa fragment

The 600 kDa fragment is shown in panel A. α_2 M-CH₃NH₂ is shown in panel B. The bar represents 25 nm.

Association of α_2 M-CH₃NH₂ and 600 kDa fragment with hepatocytes

Previous investigators have demonstrated that the 600 kDa fragment does not interact with the α_2 M receptor [17,19]. As an additional control for our preparation, ¹²⁵I- α_2 M-CH₃NH₂

(1.0 nM) was incubated with hepatocytes in the presence of α_2 M-CH₃NH₂ or the 600 kDa fragment. No decrease in cell-associated radioactivity was detected with the 600 kDa fragment at concentrations up to 200 nM after incubation for 3 h at 37 °C. Under equivalent conditions, 200 nM- α_2 M-CH₃NH₂ decreased cell-associated radioactivity by 70 %.

Fig. 3. High-magnification composite of α_2 M-CH₃NH₂ and the 600 kDa fragment

Molecules were selected based on apparent similarity in orientation relative to carbon film and the electron beam. α_2 M-CH₃NH₂ is shown in row A. The 600 kDa fragment is shown in row B. These representative examples were selected from three different preparations. The bar represents 25 nm.

Fig. 4. Scale diagrams of $\alpha_2\text{M-CH}_3\text{NH}_2$ and the 600 kDa fragment

Easily identified molecular landmarks were utilized to measure intramolecular distances in $\alpha_2\text{M-CH}_3\text{NH}_2$ and the 600 kDa fragment. The letters (a–e) demonstrate the measurements made on either form of $\alpha_2\text{M}$. These letters correspond to the parameters listed in Table 1. On the basis of the measurements presented in Table 1, the distance between paddles (parameter a) and the length of the lateral walls (parameters b and c) in both $\alpha_2\text{M-CH}_3\text{NH}_2$ and the 600 kDa fragment have been drawn to scale.

Table 1. Measurements of $\alpha_2\text{M-CH}_3\text{NH}_2$ and the 600 kDa fragment

The parameters measured are those described in Fig. 4. Note 1 Å = 0.1 nm (SI unit).

Parameter	Value	
	$\alpha_2\text{M-CH}_3\text{NH}_2$	600 kDa fragment
a	133.9 ± 8.0 Å	136.4 ± 8.5 Å
$\frac{1}{2}(b+c)$	191.7 ± 7.1 Å	154.5 ± 9.2 Å
$\frac{1}{2}(d+e)$	212.0 ± 8.5 Å	181.9 ± 6.9 Å
$\frac{1}{2}(b+c)/a$	1.44 ± 0.11	1.13 ± 0.08
$\frac{1}{2}(d+e)/a$	1.59 ± 0.14	1.34 ± 0.11

Electron-microscopy studies

Papain-treated $\alpha_2\text{M-CH}_3\text{NH}_2$ retained many of the characteristics of the parent molecule, as determined by electron microscopy (Fig. 2). The prominent lateral walls were still present and readily identified when the $\alpha_2\text{M}$ was properly oriented. The paddle structures in the centre of the hypothetical 'hollow cylinder' appeared unchanged.

Many of the 600 kDa molecules oriented on the carbon film so that the length of the cylinder was parallel to the electron beam (arrows in Fig. 2, panel A). The resulting image was an irregular 'doughnut'. Close examination revealed that the 'doughnut-like' image was probably formed by the two lateral walls and the paddle structures (the external structure of the cylinder).

When papain-treated and untreated $\alpha_2\text{M-CH}_3\text{NH}_2$ were oriented comparably, the lateral walls in the 600 kDa fragment were significantly shorter. This is most easily appreciated in the high-magnification composite shown in Fig. 3. In control experiments, $\alpha_2\text{M-CH}_3\text{NH}_2$ was treated with 5.0 mM-iodocetamide for 30 min (no papain). The alkylating agent did

not affect the image of $\alpha_2\text{M-CH}_3\text{NH}_2$ in negatively stained preparations.

Measurements were made on 25 views each of $\alpha_2\text{M-CH}_3\text{NH}_2$ and the 600 kDa fragment as shown diagrammatically in Fig. 4. The distance between the paddles in each molecule was determined to control for differences in projection (molecular angle relative to electron beam). For both the 600 kDa fragment and $\alpha_2\text{M-CH}_3\text{NH}_2$, the average distance between paddles was equivalent [≈ 13.5 nm (135 Å)]. By contrast, the lateral arms in the 600 kDa fragment were decreased in length by 19% [≈ 3.7 nm (37 Å)], $P < 0.001$. Table 1 summarizes the measurements made with $\alpha_2\text{M-CH}_3\text{NH}_2$ and the 600 kDa fragment. When the average wall length in each molecule was divided by the distance between paddles, the 600 kDa fragment and $\alpha_2\text{M-CH}_3\text{NH}_2$ remained significantly different ($P < 0.001$). The models shown in Fig. 4 have been drawn to scale on the basis of the measurements listed in Table 1.

DISCUSSION

The $\alpha_2\text{M}$ receptor recognizes $\alpha_2\text{M-CH}_3\text{NH}_2$ and $\alpha_2\text{M}$ -proteinase complexes, but not native (slow form) $\alpha_2\text{M}$ [7,10]. Digestion of $\alpha_2\text{M-CH}_3\text{NH}_2$ with papain under acidic conditions causes the dissociation of the inhibitor into 600 kDa and 18 kDa fragments [17]. It has been proposed that the 18 kDa fragment includes the region that is recognized by the $\alpha_2\text{M}$ receptor [17,18]. The 600 kDa fragment retains no affinity for the receptor [17,19]. On the basis of immunoelectron-microscopic studies [20], the 18 kDa fragments should be located near the four apices of the lateral walls. It is also possible that the 600 kDa fragment loses receptor affinity because of irreversible denaturation during papain digestion.

The present study confirms and extends previous investigations of the $\alpha_2\text{M}$ receptor-binding domains by providing images of the 600 kDa fragment. The following conclusions are offered. After papain treatment, the basic structure of the transformed or fast-form $\alpha_2\text{M}$ remains intact. This structure is most simply described as a hollow cylinder. The body of the cylinder is composed of the two lateral walls that extend the total length of the molecule. The lateral walls do not meet to form a uniform cylinder shape. In the gaps between the walls, the major stain-excluding structures are the paddles. With proper orientation, bound proteinases may be viewed centrally through the gaps in the walls, overlapping the paddles [15,16].

As might be predicted by previous immunoelectron-microscopic studies [20], the lateral walls in the 600 kDa fragment are significantly decreased in length. In other words, the epitopes in $\alpha_2\text{M-CH}_3\text{NH}_2$ recognized by 'receptor recognition site-specific' monoclonal antibodies are absent after papain digestion. Regions of $\alpha_2\text{M-CH}_3\text{NH}_2$ that are preserved in the 600 kDa fragment probably do not contribute affinity to the receptor-binding interaction. Most notably, the paddle structures are not part of the 18 kDa fragments and probably do not bind to the receptor.

The f.p.l.c. data and electron micrographs presented here suggest that the molecular radius (apparent Stokes radius) of papain-treated $\alpha_2\text{M-CH}_3\text{NH}_2$ is decreased compared with the parent molecule. We propose that the change in molecular shape may explain the heterogeneity in orientation of the 600 kDa fragment on the carbon films. After papain digestion, many of the molecules formed images that resembled doughnuts. This structure should not be confused with the 'doughnut-like' images described for native or slow-form $\alpha_2\text{M}$ [11–14]. The structure of native $\alpha_2\text{M}$ remains unclear; however, it is unlikely that the native $\alpha_2\text{M}$ doughnut is a cylinder oriented parallel to the electron beam, as described for the 600 kDa fragment in this study. Therefore the similar images obtained in some electron

micrographs of native α_2 M and some projections of the 600 kDa fragment probably represent very different structures.

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