Crystallisation and preliminary X-ray analysis of the receptor-binding domain of human and bovine α_2-macroglobulin

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Abstract **The receptor-binding domains (RBDs) of human and bovine α_2-macroglobulin (α_2M) have been isolated after limited proteolysis of methylamine-treated α_2M with papain. Single crystals of the RBDs have been grown by vapour diffusion. Crystals of human RBD are very thin plates unsuited for data collection. However, crystals of RBD from bovine α_2M give diffraction patterns suitable for X-ray analysis, and a complete dataset with a maximum resolution of 2.3 Å has been collected with synchrotron radiation at cryogenic temperature. The crystals belong to spacegroup $P3_121$ or $P3_221$ with cell parameters $a = b = 106.8$ Å, $c = 72.2$ Å.**

Key words: α-Macroglobulin; Receptor-binding; Crystallization

1. Introduction

The α-macroglobulins constitute a large family of homologous high molecular mass protease inhibitors in the blood of vertebrates and invertebrates, and in the egg white of birds and reptiles (for a short review, see [1]). Human and bovine α_2-macroglobulin (α_2M) are homotetramers of 180-kDa subunits. Two subunits are disulphide-bridged in an antiparallel arrangement, and two such dimers are noncovalently assembled to tetramers [2]. Proteolytic cleavage in the so-called 'bait region' (residues 667–705 in human α_2M [3]) triggers a conformational change that entraps the protease within the α_2M tetramer. This change in conformation activates the internal β-Cys-γ-Gln thiol esters, preferentially giving rise to ε-Lys (protease) -γ-Glu (α_2M) cross-links [4–6]. In human, but not in bovine α_2M [7], the conformational changes can also be induced by cleavage of the thiol ester with small nucleophiles such as methylamine [8] without cleavage of the bait region. The α_2M-protease complexes and α_2M-methylamine (α_2M-MA) are rapidly cleared from the circulation [9,10] through binding of the exposed C-terminal domain (RBD) [11] to an endocytosing receptor known as α_2M receptor/low density lipoprotein receptor-related protein (α_2MR/LRP) [12–14]. Electron microscopic studies [15,16] have shown that the receptor-binding domains of human α_2M are located at the tips of the H-like structure. Recently, it has been shown by X-ray crystallography that human α_2M-MA has an almost spherical shape from which four minor bodies protrude. These bodies probably represent the RBDs [17].

Here, we report the crystallisation and preliminary X-ray analysis of the RBDs isolated from human and bovine α_2M.

2. Materials and methods

Sepharose 4B, Con A Sepharose, Mono S, DEAE Sephacel and Sephacryl S-300 HR were from Pharmacia, crystalline papain was from Boehringer Mannheim, and standard chemicals were from Merck or Sigma. ProBlott membranes and sequencer chemicals were from Applied Biosystems. Zn^{2+}-chelate Sepharose and lysine Sepharose were prepared from Sepharose 4B as described by the manufacturer. Outdated human plasma was obtained from the Århus University Hospital Bloodbank, and bovine blood was from the local slaughterhouse.

Human α_2M was prepared as described [4]. Bovine α_2M was purified as follows: Plasmin and plasminogen were removed by stirring bovine plasma with lysine Sepharose. After precipitation with PEG 6000, the 4–12% fraction was loaded on a DEAE Sephacel column equilibrated with 10 mM sodium phosphate (NaP), 10 mM NaCl, pH 7.4, and eluted with a linear gradient from 10 to 300 mM NaCl. The α_2M-containing fractions were loaded on the Zn^{2+}-chelate Sepharose column, washed with 20 mM NaP, 300 mM NaCl, pH 7.4, and eluted with 100 mM sodium acetate, pH 5.0. After gel filtration on Sephacryl S-300 HR in 100 mM NaP, pH 8.0, the preparation was homogenous in SDS-PAGE. To cleave the thiol esters, human and bovine α_2M were treated with 200 mM methylamine and 20 mM iodoacetamide for 4 h [4]. The receptor-binding domains were obtained after cleavage of α_2M-MA with papain as described [18].

To separate differently glycosylated variants of human RBD, the material was loaded on a column of Con A Sepharose, equilibrated with 20 mM Tris-HCl, 0.5 M NaCl, pH 7.4, and the bound material was eluted by including 0.5 M αD-methylmannopyranoside in the equilibration buffer. The non-binding and the binding fractions were pooled separately, and each pool was loaded on a Mono S column equilibrated with 10 mM ammonium acetate, pH 4.5, after dialysis against the same buffer. The material was eluted with a linear 0–0.5 M gradient of ammonium acetate, pH 5.0. The non-Con A-binding fraction eluted as four peaks, and the Con A-binding fraction as two peaks. The latter material was used for crystallisation trials because it was homogenous in SDS-PAGE (not shown).

Amino acid analysis was performed as described [19], after hydrolysis with 6 M HCl, 0.1% phenol and 5% thioglycolic acid at 110 °C for 16 h. Excised bands from the ProBlott membrane was wetted with methanol prior to hydrolysis [20]. The values of Ser and Thr were corrected for a loss of 10 and 5%, respectively. To determine the amount of aminosugar, hydrolysis was performed as described above, but only for 3 h. The values of $GlcNH_2$ was corrected for a loss of 5%.

N-terminal sequencing of the RBDs was performed with an Applied

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Abbreviations: α_2M, α_2-macroglobulin; α_2M-MA, methylamine-treated α_2-macroglobulin; α_2MR/LRP, α_2M receptor/low density lipoprotein receptor-related protein; β-OGP, 1-*O*-*n*-Octyl-β-D-glucopyranoside; Con A, Concanavalin A; $GlcNH_2$, glucosamine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); Mes, 2-(*N*-morpholino)-ethanesulfonic acid; NaP, Sodium phosphate; PEG, Polyethylene glycol; RBD, Receptor-binding domain of α_2M (approx. residues 1314–1451); SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; Tris, Tris(hydroxymethyl)amino-methane.

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Biosystems 477A sequencer equipped with an on line 120A HPLC, using standard protocols. An in vitro competition assay [21] was used to determine the receptor-binding activity of bovine RBD.

Protein used for crystallisation was concentrated to ~10 mg/ml in Centricon-10 cells (Amicon) and flash frozen in liquid nitrogen. Conditions for crystallisation of RBD were investigated by repeated use of the incomplete factorial approach [22]. The variables screened were precipitant, pH, temperature, ionic strength, mono- and divalent cations, final protein concentration and additives (alcohols and β-OGP). Vapour diffusion experiments were performed in Cryschem trays using a crystallisation robot (G.R. Andersen and J. Nyborg, submitted).

Data were collected at cryogenic temperature with a MAR-imaging plate using synchrotron radiation, λ = 1.488 Å, at beamline 7–2, SRS, Daresbury Laboratory, UK. Prior to mounting by the loop method [23] and flash-freezing at 100 K in a stream of nitrogen, crystals were gradually transferred to a cryoprotective solution containing 26% PEG 6000, 7% glycerol, 25 mM $CaCl_2$, pH 6.9–7.4. The density of the crystals was determined in a Ficoll gradient [24]. The program DENZO [25] was used for indexing and integration of data. Programs from the CCP4-package [26] were used for scaling, postrefinement and merging.

3. Results and discussion

3.1. Isoforms of RBD

The isolated human RBD appeared as two bands of ~22 kDa and one faint band at ~15 kDa in reducing SDS-PAGE (not shown) in agreement with earlier results [11,18]. Sequence analysis of the individual bands revealed only one N-terminal sequence (Fig. 1). After electroblotting onto a ProBlott membrane, each band was excised and its composition determined. The amino acid compositions were as expected for residues 1314–1451 of human α_2M [27]. The two bands at 22 kDa were found to contain 5.0 mol $GlcNH_2$/mol RBD, indicating full glycosylation of its single site [27], whereas the 15-kDa band contained none, indicating no glycosylation.

The isolated RBD from bovine α_2M appeared as three separate bands (~26, 25 and 22 kDa) in reducing SDS-PAGE (not shown). In contrast to human RBD, two N-terminal sequences were found when the pool was subjected to sequence analysis. As shown in Fig. 1, 80–90% of the material (form 1) had the sequence previously determined [28], while the remainder (form 2) included 6 upstream residues. The amino acid composition of each variant was in good agreement with that expected from the known sequence of bovine RBD, corresponding to residues 1313–1451 of human α_2M [28]. The 26- and 25-kDa variants were found to contain 7.1 and 6.3 mol $GlcNH_2$/mol protein, respectively, indicating glycosylation of both putative sites [28]. The 22-kDa variant contained 4.2 mol $GlcNH_2$/mol protein, indicating glycosylation of (in average) only one site.

Bovine RBD was found to bind to α_2MR/LRP [21] with a K_d of 60 nM, in agreement with provious determinations [28].

3.2. Crystallisation

The Con A-binding fraction of human RBD crystallised in 7–10 days at 30–33% PEG 6000, 100 mM KCl, pH 4.0–4.2, at 20 °C. Even the best crystals were thin (~1.05 × 0.75 × 0.02 mm^3) and appeared to consist of layers of very thin plates. Crystals were also obtained when $CaCl_2$ or $MgCl_2$ were included in place of KCl, but these crystals were smaller and more irregular than those obtained with KCl.

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Human α2M:          (1301)LQTSLKYNIL PEKEEFPFAL GVQTLPQTCD
Human RBD:                          EEFPFAL
Bovine RBD form 1:                  KDEFPFAL E
Bovine RBD form 2                YNIL PKKDE
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Fig. 1. Sequence alignment of bovine RBD with human α_2M and RBD. Two different N-terminal sequences of bovine RBD are present. The amount of form 2 was 10–20% of the amount of form 1 in different preparations.

Fig. 2. Hexagonal crystals of bovine RBD with a maximum size of 0.4 × 0.2 × 0.2 mm^3. The crystals grew by vapour diffusion in sitting drops.

Hexagonal crystals of bovine RBD (pool of the 26-, 25- and 22-kDa variants) appeared in 20 days at 28% w/v PEG 6000, pH 5.5–7.4 (100 mM Mes or Hepes titrated with Tris), and 25–125 mM $CaCl_2$. Crystals were obtained at both 4°C and 20 °C with a maximum size of 0.2 × 0.2 × 0.4 mm^3 (Fig. 2). The presence of Ca^{2+}, although not strictly necessary, considerably promoted crystal growth. Calcium could not be substituted by zinc, nickel, cadmium or magnesium.

3.3. X-ray analysis

Upon mounting in glass capillaries, the thin crystals of human RBD bent, resulting in smeared reflections. During a preliminary exposure at the DESY synchotron, EMBL outstation, Hamburg, the crystals showed diffraction to 2.0 Å, and the cell parameters were estimated to be a = 47 Å, b = 65 Å and c = 122 Å. However, the maximum resolution varied, depending on the orientation of the crystals. Despite considerable efforts, the quality of the RBD crystals could not be improved. Recombinant human RBDv (residues 1299–1451) [29] also crystallised readily, but these crystals did not appear to have better properties than those of the authentic protein.

At 4°C crystals of bovine RBD mounted in capillaries ceased to diffract X-rays in a few minutes. However, the crystals diffracted X-rays to high resolution when exposed to synchrotron radiation at cryogenic temperature. Oscillation images of 1.5° with useful reflections extending to 2.3 Å were collected within 3–5 min of exposure (Fig. 3). The crystals belong to space group $P3_121$ or $P3_221$ as determined from systematic extinctions and symmetry in the diffraction patterns. The cell parameters were determined to be $a = b$ = 106.8 Å, c = 72.2 Å. The mosaicity of the best crystal was ~0.4°. A complete native dataset extending to 2.3 Å with a R_{sym} value of 6.1% (25.3% in the range 2.42–2.30 Å) was obtained from scaling and merging of data from a single crystal.

Using a 18–37% Ficoll gradient (ρ = 1.06–1.14 g/cm^3) crystals of bovine RBD were estimated to have a density of 1.13

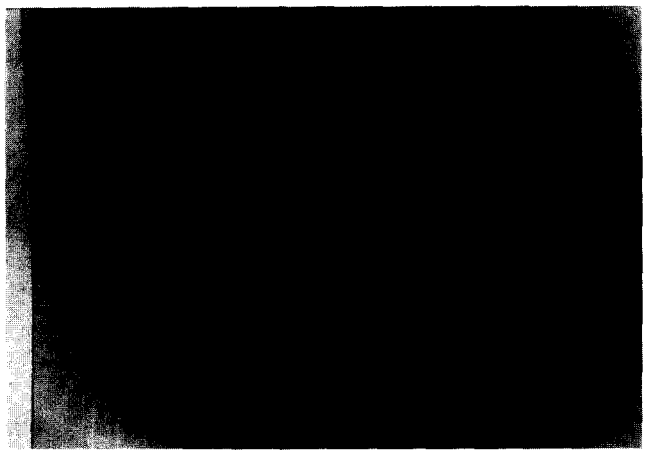

Fig. 3. Oscillation image of 1.5° obtained from exposure to synchrotron radiation for 180 s. The temperature during the exposure was 103 K. The image was recorded at beamline 7.2, SRS, Daresbury Laboratory, UK.

g/cm^3. According to [24], the theoretical density in this system is 1.13 and 1.20 g/cm^3, respectively, for two and three molecules of bovine RBD/asymmetric unit.

From the density measurement, the crystals of bovine RBD probably contain two molecules/asymmetric unit assuming a combined size of protein and carbohydrate of ~20 kDa, and they have a solvent content of 63%. This results in a value of V_m = 3.30 Å^3/Da, which is within the usual range of 1.68–3.53 Å^3/Da normally observed for protein crystals [30].

The K_d for binding of human and bovine RBD to α_2MR/LRP, ~60–125 nM in different systems [11,18,28], is much higher than the K_d for binding intact α_2M-MA (~40 pM and 2 nM for the high- and low-affinity binding, respectively) [21]. Therefore, it has been discussed whether RBD in its structure contains all information necessary for receptor recognition by α_2M [28,31]. However, the finding that human RBDv, which includes 12 additional upstream residues, binds to α_2MR/LRP with a K_d of ~8 nM [29] indicates that RBD constitutes the receptor-binding domain of α_2M, but its conformation may by subtly affected by neighbouring residues.

The ability of human and bovine RBD to readily crystallise clearly suggests that this domain has a well-defined tertiary structure. The high resolution of the X-ray diffraction of bovine RBD will allow the first detailed structure of a functionally important α_2M domain to be determined, and that structure will provide a framework for understanding the interaction of the multifunctional α_2MR/LRP [32] with its ligands.

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