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Crystallization and preliminary X-ray analysis of methylamine-treated α_2-macroglobulin and 3 α_2-macroglobulin–proteinase complexes

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Crystals of methylamine-treated α_2-macroglobulin (α_2M–MA), α_2-macroglobulin in complex with two molecules of trypsin, α_2M–T2, one molecule of plasmin, α_2M–PL, and one molecule of plasmin followed by methylamine-treatment, α_2M–PL(MA), have reproducibly been obtained using ammonium sulfate or magnesium sulfate as precipitants. The crystals are fragile tetragonal bipyramids of up to 1.5 mm in length. Crystals of α_2M–MA diffracted to at least 9 Å resolution, crystals of α_2M–T2 diffracted to 10 Å resolution and crystals of α_2M–PL and α_2M–PL(MA) diffracted to 11 Å resolution. For α_2M–MA the cell parameters were determined as: $a=b=257$ Å, $c=555$ Å; and for α_2M–T2 as: $a=b=247$ Å, $c=559$ Å. For both preparations the space group was I4(1)22. As estimated from density measurements, the crystals of α_2M–MA and α_2M–T2 contain one 360 kDa α_2M dimer per asymmetric unit. The volume of the asymmetric unit/molecular weight, V_m, was estimated at 5.6 Å^3/Da. The crystal parameters of α_2M–PL and α_2M–PL(MA) were not determined.

α_2-Macroglobulin; Proteinase inhibitor; Crystallization

1. INTRODUCTION

The plasma proteinase inhibitor α_2-macroglobulin (α_2M) is a glycoprotein composed of 4 identical 180 kDa subunits. Two subunits are disulfide-bridged, forming 360 kDa dimers; two such dimers are noncovalently assembled to a 720 kDa tetramer (for a recent short review on α-macroglobulins see [1]). Each subunit contains an activation cleavage region (the 'bait' region) and an internal β-Cys–γ-Glu thiol ester. To initiate complex formation a proteinase must cleave at least one bait region. This results in a conformational change which leads to 'entrapment' of the proteinase. Typically α_2M binds and inhibits 2 molecules of proteinases the size of e.g. trypsin, but only 1 molecule of the larger plasmin. During complex formation the thiol esters are cleaved and the bound proteinase becomes cross-linked to α_2M primarily through amide cross-links. This involves several Lys-residues of the proteinase and the Glx-moieties of the thiol esters [2]. The thiol esters can also be cleaved by amines such as methylamine, leading to inactivation of α_2M. In both processes Cys–SH groups appear. In electron micrographs native α_2M resembles irregular donuts [3–6]. After reaction with proteinases or methylamine, α_2M has a more compact H-like shape with the proteinase(s) occupying a central elongated cavity [5–10]. From electron microscopy studies several low-resolution models of native and methylamine- or proteinase-treated α_2M have been proposed [3–11].

Although crystals of various forms of α_2M were obtained earlier no, or only modest, X-ray diffraction was observed [12,13]. We report here the crystallization of different forms of α_2M. In the case of methylamine-treated α_2M diffraction data extending to at least 9 Å resolution can be obtained.

Abbreviations: α_2M, α_2-macroglobulin; α_2M–MA, methylamine-treated α_2-macroglobulin; α_2M–T2, α_2-macroglobulin–trypsin complex (1:2); DPDS, 2,2′-dipyridyldisulfide; α_2M–PL, α_2-macroglobulin–plasmin complex (1:1); CTA, committee of thrombolytic agents; α_2M–PL(MA), α_2-macroglobulin–plasmin complex (1:1) further treated with methylamine; DFP, diisopropylfluorophosphate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid.

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2. MATERIALS AND METHODS

α_2M was prepared from pooled outdated human plasma [14]. Four preparations were used in the present experiments, which were 85–95% active in terms of titrable SH-groups after reaction with methylamine. Plasminogen was prepared by lysine-Sepharose chromatography [15]. Urokinase was obtained from Abbott (Chicago, USA). Trypsin was from Boehringer (Mannheim, Germany).

α_2M–MA was obtained after incubating α_2M (10 mg/ml) with 0.2 M methylamine at pH 8.0 in the presence of 10 mM iodoacetamide for 2 h. α_2M–T2 was prepared by incubation with a 4-fold excess of trypsin in the presence of 0.1 M benzamidine to decrease the concentration of free trypsin [16]. SH-groups were blocked by reaction with 1 mM DPDS. α_2M–PL was prepared by treating plasminogen with urokinase (800 CTA units/mg plasminogen) in the presence of equimolar amounts of α_2M. SH-groups were blocked by reaction with 1 mM DPDS. Since only approx. half of the bait regions and the thiol esters were cleaved by reaction with plasmin, in one experiment α_2M–PL was further treated with methylamine and DPDS to cleave remaining thiol esters and generate α_2M–PL(MA). After complex formation 10 mM

DFP was added to inactive the bound proteinase, and the different forms of α_2M were recovered by gel chromatography on columns of Sephacryl S-200 equilibrated and eluted with 20 mM Tris-HCl, pH 7.7. The preparations were concentrated to approx. 10–14 mg/ml before use. All experiments were performed at room temperature.

Crystals were grown by vapor diffusion [17] in 8–40 μl hanging- or sitting-drops using solutions containing approx. 10 mg/ml of α_2M (final concentrations) and 1 mM DFP. Density measurements were made in a gradient of water-saturated *o*-xylene and bromobenzene [18] and in a Ficoll gradient [19]. X-ray oscillation films were recorded using synchrotron radiation at LURE, Paris. Cell parameters were determined and refined by the auto-indexing program REFIX [20].

3. RESULTS

Crystals of α_2M–MA and α_2M in complex with trypsin and plasmin were grown using 20–30% saturated ammonium sulfate or 1.3–1.5 M magnesium sulfate buffered at pH 6.5–8.0 as precipitants. All experiments were performed at 20°C. Crystals appeared in 1–4 weeks, and growth continued for several months (Table I). All crystals were fragile, elongated tetragonal bipyramids as shown in Fig. 1. In the crystallization droplets the crystals of α_2M–T2 deteriorated after a few weeks while crystals of the other 3 α_2M-species were stable for several months.

In reducing-SDS-PAGE re-dissolved crystals showed the characteristic band patterns of α_2M–MA (180 kDa), α_2M–T2 (85 kDa and cross-linked products of larger sizes), α_2M–PL and α_2M–PL(MA) (85, 180 kDa and cross-linked products) (not shown).

When exposed to synchrotron radiation crystals of α_2M–MA and all preparations of α_2M–proteinase complexes showed diffraction. Crystals of α_2M–MA diffracted to at least 9 Å resolution, crystals of α_2M–T2 diffracted to 10 Å resolution, and crystals of α_2M–PL and

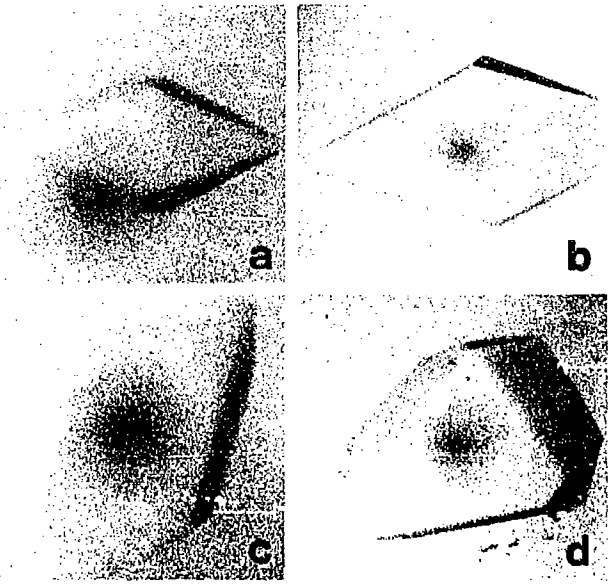

Fig. 1. Crystals of (a) α_2M–MA, (b) α_2M–T2, (c) α_2M–PL and (d) α_2M–PL(MA). The maximal dimension is 1.5 mm. The crystals grew reproducibly in 1–4 weeks by vapor diffusion in hanging- or sitting-drops.

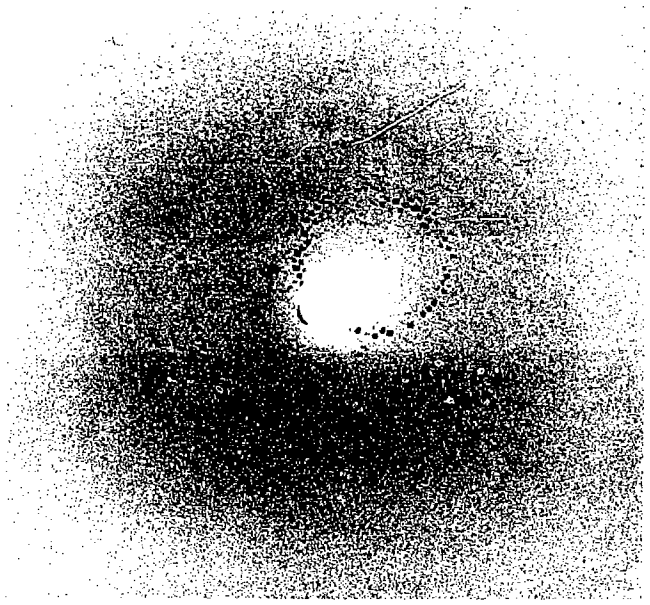

Fig. 2. A 0.5° rotation image obtained after 16.6 min exposure to synchrotron radiation of a crystal of α_2M–MA. The crystal-to-film distance was 149 mm and λ=1.4 Å. The anisotropic background was seen with crystals of all 4 preparations. The magnification is approx. ×1.35.

α_2M–PL(MA) diffracted to 11 Å resolution. For all crystals decay was significant following 15–30 min of exposure, and was slowest for α_2M–MA. An example of the diffraction of a crystal of α_2M–MA is given in Fig. 2. An anisotropic background extending to the maximal resolution was observed for all crystals.

For α_2M–MA the cell parameters were determined as: $a=b$=257 Å, c=555 Å; and for α_2M–T2 as: $a=b$=247 Å, c=559 Å. For both preparations the space group was

Table I

α_2M Complexes

	α_2M–2T	α_2M–MA	α_2M–PL	α_2M–PL (MA)
Precipitant	20–25% $(NH_4)_2SO_4$ pH 7.0–8.0 or 1.5 M $MgSO_4$ pH 6.5–7.5	20–30% $(NH_4)_2SO_4$ pH 7.3–7.8	1.3–1.5 M $MgSO_4$ pH 6.75–7.25	28–30% $(NH_4)_2SO_4$ pH 6.5–7.5
Additive	–	0.15–1.5% β- octyl-glucoside	–	–
Buffer	Tris-HCl	Tris-HCl	Tris-HCl MOPS	Tris-HCl MES
Dimensions	1.5×0.8×0.5 mm	1.5×0.8×0.5 mm	1.0×0.6×0.3 mm	1.2×0.8×0.5 mm

Final concentrations of precipitants are shown.

I4(1)22 as determined from systematic extinctions and symmetry in the diffraction patterns. The *a*- and *b*-axes are along the edges in the equitorial plane, and the *c*-axis is along the pyramidal axis in the crystals. The crystal parameters of α_2M–PL and α_2M–ML(MA) were not determined.

Crystals of α_2M–T2 had a density of 1.10 ± 0.01 g/cm^3 using the *o*-xylene bromobenzene method, and the density of the reservoir was 1.07 g/cm^3. Assuming, according to the model in [18], that the weight fraction of protein-bound water is 0.25, and, further, that the free solvent has a density equal to that of the reservoir, the theoretical density of the crystal would be 1.13 g/cm^3 for one dimer plus one bound trypsin molecule (385 kDa) per asymmetric unit. For two dimers per asymmetric unit the theoretical density would be 1.18 g/cm^3.

In the Ficoll gradient the density of the crystals was 1.06 ± 0.01 g/cm^3. Using the model of [19] the theoretical density in this system is 1.08 g/cm^3 and 1.15 g/cm^3 for one dimer and two dimers, respectively, per asymmetric unit.

4. DISCUSSION

α_2M–MA and α_2M complexes with trypsin and plasmin reproducibly form similar bipyramidal crystals using ammonium sulfate and magnesium sulfate as precipitants. The cell parameters of crystals of α_2M–MA and α_2M–T2 are very similar. These features are in general accord with the observations that (i) proteinases, despite having widely different sizes, are largely buried within the α_2M structure [3–11], and (ii) that α_2M–MA and α_2M–proteinase complexes, in terms of overall hydrodynamic and electrophoretic properties [21–23] and receptor recognition [24], are very similar. However, immunological procedures reveal subtle differences in the conformations of α_2M–MA and α_2M–proteinase complexes [5].

From the density measurements, the crystals of α_2M–2T (and of α_2M–MA) probably contain one 360 kDa dimer rather than two dimers per asymmetric unit. However, the estimated value of V_m=5.6 Å^3/Da for α_2M–T2 is above the usual range of 1.68–3.53 Å^3/Da [25]. This could explain the extreme softness of the crystals. Considering the size of the well-formed crystals the limited resolution of their diffraction is surprising. This is likely to reflect local disorder in the crystal packing, perhaps due to slightly different conformations of segments of the subunits of individual α_2M tetramers. This may be particularly important for α_2M–proteinase complexes, where individual proteinase molecules are located in the binding cavity in slightly different orientations relative to the α_2M bulk structure [2].

Tetragonal bipyramidal crystals of α_2M–trypsin grown under similar conditions were reported earlier, but they showed very little diffraction [12]. The cause of this is not known, but recently crystals of α_2M–MA belonging to another crystal form were grown from polyethylene glycol solutions; these crystals diffracted to about 10 Å resolution [13].

To possibly improve the quality of the crystals the homogeneity of the starting material(s) must be increased. In this respect it will be important to use α_2-macroglobulin isolated from the plasma of individual donors, and highly purified proteinases.

Despite the relatively low resolution of the present crystals they are suitable for obtaining more accurate structural information on methylamine-treated and proteinase-complexed α_2M than has previously been provided by electron microscopy.

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