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Cloning, purification, crystallization and preliminary X-ray studies of human α_1-microglobulin

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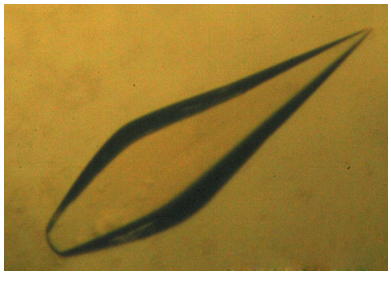

α_1-Microglobulin (α_1m) is one of the phylogenetically most widespread lipocalins and is distributed in various organs and tissues, including liver, heart, eye, kidney, brain, lung, pancreas and skeletal muscle. α_1m has been found to exert multifarious functions, including interacting with IgA, albumin and prothrombin, binding strongly to haem and exhibiting reductase activity. Nevertheless, little structural information is available regarding these functions of α_1m. Since determination of three-dimensional structure is a powerful means of functional characterization, X-ray crystallography was used to accomplish this task. Here, the expression, purification, crystallization and preliminary crystallographic analysis of human α_1m are reported. The crystal belonged to space group $P4_3$, with unit-cell parameters $a = b = 36.45$, $c = 112.68$ Å, and diffracted to a resolution of 2.0 Å. The crystals are most likely to contain one molecule in the asymmetric unit, with a V_M value of 1.63 Å^3 Da^{-1}.

1. Introduction

The lipocalins, which are found in animals, plants and bacteria, are a superfamily of proteins consisting of 30–35 members (Flower, 1996; Åkerström *et al.*, 2000). The family has a well conserved three-dimensional structure, folding into a barrel consisting of eight anti-parallel β-strands with a closed end and an open end which provides a binding site for small hydrophobic ligands. Interestingly, lipocalins exert a surprisingly wide array of biological functions, but only a few members have been characterized, including the plant enzyme violaxanthin de-epoxygenase (Hieber *et al.*, 2000), insect bilin-binding protein (Huber *et al.*, 1987), and prostaglandin D-synthase (Urade & Hayaishi, 2000) and α_1-microglobulin (α_1m) from animals (Åkerström & Lögdberg, 2006).

α_1m, also called protein HC, is one of the phylogenetically most widespread lipocalins (Tejler & Grubb, 1976). To date, it has been found in mammals, birds, fish and amphibians (Åkerström & Lögdberg, 2006). α_1m is mainly synthesized in the liver, secreted into the blood and rapidly distributed to various organs and tissues, including liver, heart, eye, kidney, brain, lung, pancreas, skeletal muscle and blood. The lipocalin exits in a free form or as a component of protein complexes by binding to IgA, albumin and prothrombin (Berggård *et al.*, 1998; Allhorn *et al.*, 2002). The amino-acid sequence is highly conserved and the extent of its distribution indicates that α_1m plays an important physiological function. Previous research has found that α_1m can bind strongly to haem and that t-α_1m, a truncated variant α_1m that lacks the C-terminal tetrapeptide LIPR, can degrade haem with the help of Hb (Allhorn *et al.*, 2002). In addition, the secreted protein also exerts reductase and antioxidant activities by serving as a radical scavenger (Åkerström *et al.*, 2007). Nevertheless, little information is available about the active sites (or regions) and the mechanisms of the reductase and antioxidant activities of α_1m. In order to further investigate the biochemical and physiological functions of α_1m, we report the expression, purification, crystallization and preliminary X-ray crystallographic studies of human α_1m. Structure determination will be pursued using experimental phasing methods. Determination of the three-dimensional structure of α_1m will help in understanding how this widespread protein exerts its multitudinous functions.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	1.0
Space group	$P4_3$
Unit-cell parameters (Å)	$a = b = 36.45$, $c = 112.68$
Resolution range (Å)	50–2.0 (2.07–2.00)
Total reflections	284280 (1946)
Unique reflections	9947
Completeness (%)	99.9 (98.9)
$\langle I/\sigma(I)\rangle$	16.7 (8.5)
Multiplicity	7.1 (7.3)
$R_{\rm merge}$†	7.0 (35.8)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i |I_i(hkl)|$, where $I_i(hkl)$ is the intensity of the ith measurement of an equivalent reflection with indices hkl.

2. Materials and methods

2.1. Cloning and expression

The gene encoding α_1m was amplified from human cDNA by PCR using primers (α_1mFwd and α_1mRev) that contained *Eco*RI and *Xho*I restriction sites, respectively. Both the PCR products and the plasmid pET28a were digested with *Eco*RI and *Xho*I restriction enzymes. The ligation mixture was transformed into chemically competent *Escherichia coli* DH5α cells and insertion was verified by PCR using T7 promoter and T7 terminator primers. The identity of the insert was further confirmed by DNA-sequence analysis. The final construct (pET28a-α_1m) encodes α_1m protein with an N-terminal His tag. The recombinant expression vector was transformed into chemically competent *E. coli* BL21 (DE3) cells for expression. Cultures of bacteria carrying pET28a-α_1m were grown overnight in 20 ml LB medium supplemented with 50 mg l^{-1} kanamycin. The bacteria were then used to inoculate 2.0 l LB medium and cultured at 310 K for about 4 h. The protein was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG; 0.3 m*M*) when the $OD_{600\ nm}$ reached 0.4; the culture was allowed to grow at 293 K for a further 15 h before the cells were harvested by centrifugation.

2.2. Purification

The cells were resuspended in lysis buffer (20 m*M* Tris–HCl pH 8.0, 300 m*M* NaCl) and disrupted by sonication. The cell lysate was centrifuged at 15 000*g* for 30 min at 277 K and the cell debris was removed. The recombinant protein in the supernatant was applied onto a Ni^{2+}–NTA (Qiagen) column pre-equilibrated with lysis buffer. Nonspecifically bound proteins were washed from the column with 100 ml lysis buffer containing 50 m*M* imidazole. The recombinant protein was then eluted from the column with 20 ml elution buffer (20 m*M* Tris–HCl pH 8.0, 200 m*M* imidazole). The elution buffer was then loaded onto a HiTrap Q column (GE Healthcare) pre-equilibrated with 20 m*M* Tris–HCl pH 8.0 and the target protein was eluted with a linear gradient of 0–1000 m*M* NaCl in 20 m*M* Tris–HCl pH 8.0. For crystallization, fractions containing α_1m were concentrated and buffer-exchanged into the final buffer (5 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl) using a Millipore Amicon concentrator with a 10 kDa cutoff membrane. The concentration of the purified protein was 10 mg ml^{-1} and its purity was determined to be about 95% by SDS–PAGE.

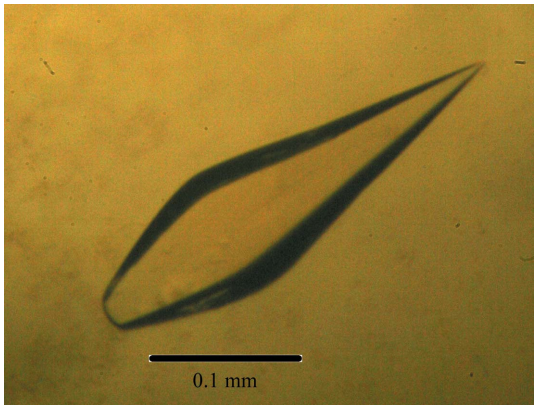

Figure 1
A crystal of human α_1m as grown by the hanging-drop method. The average dimensions of the crystals were 0.1 × 0.05 × 0.05 mm.

2.3. Crystallization

Preliminary screening of crystallization conditions was performed by the hanging-drop vapour-diffusion method with Crystal Screen, Index and PEG/Ion kits (Hampton Research). The crystallization experiments consisted of 1.0 µl protein solution mixed with 1.0 µl reservoir solution and equilibrated against 400 µl reservoir solution at 293 K. After 6 d, small crystals of α_1m were observed using a reservoir condition consisting of 20%(*w*/*v*) PEG 3350, 0.2 *M* ammonium citrate tribasic pH 7.0. Further optimization of the conditions using PEGs of different molecular weights at various concentrations gave good diffraction-quality crystals using 25–35%(*w*/*v*) PEG 3350 in the presence of 0.1 *M* HEPES pH 6.6–7.5. Crystals were obtained after 5 d equilibration against the crystallization solution and grew to full size (0.05 × 0.05 × 0.1 mm) in 15 d (Fig. 1).

2.4. Data collection and processing

Crystals were transferred to a reservoir solution adjusted to 20%(*v*/*v*) glycerol and immediately placed in a 100 K nitrogen-gas stream. X-ray diffraction data were collected from a single crystal using a MAR CCD detector on beamline U17O at SSRF (Fig. 2). The images were processed using *MOSFLM* (Leslie, 1992) and *SCALA* from the *CCP*4 suite (Evans, 2006; Winn *et al.*, 2011). The final data-collection and processing statistics are given in Table 1.

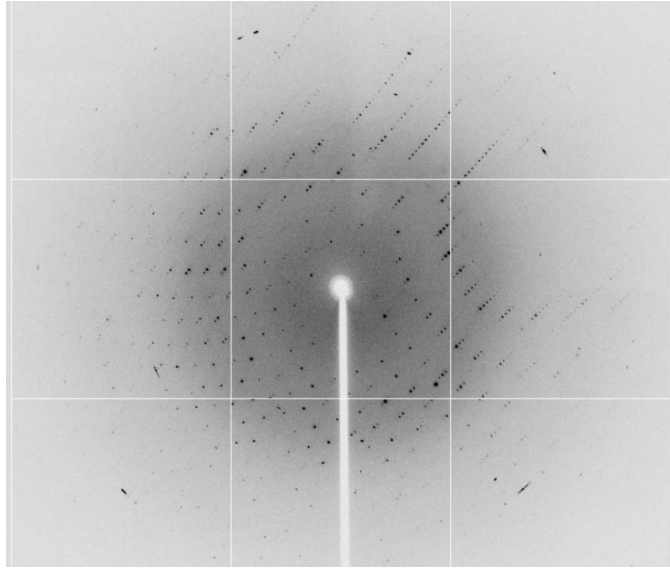

Figure 2
X-ray diffraction image from a native human α_1m crystal recorded using a MAR CCD detector. The edge of the detector corresponds to a resolution of 2.0 Å.

3. Analysis and discussion of preliminary X-ray diffraction results

The overexpression method led to 20 mg pure protein being obtained from 3 l LB culture medium after purification by metal-affinity and ion-exchange chromatography. The purified α_1m was crystallized under PEG-containing conditions. Crystals suitable for X-ray diffraction were obtained after 15 d. A total of 284 280 measured reflections were merged into 9947 unique reflections with an $R_{\rm merge}$ of 7.0%. The merged data set was 99.9% complete to 2.0 Å resolution. The relevant data-collection statistics are given in Table 1. The α_1m crystal belonged to the tetragonal space group $P4_3$, with unit-cell parameters $a = b = 36.45$, $c = 112.68$ Å (Table 1). Based on the molecular weight of α_1m (about 23 kDa) and space group $P4_3$, solvent-content analysis indicated that one molecule could be accommodated per asymmetric unit, suggesting a $V_{\rm M}$ value (Matthews, 1968) of 1.63 Å^3 Da^{-1} and a solvent content of 24.4%. Molecular replacement using *AMoRe* (Navaza, 2001), *Phaser* (McCoy *et al.*, 2005) and *MOLREP* (Vagin & Teplyakov, 2010) was carried out using the human prostaglandin D-synthase structure (PDB entry 3o19; Zhou *et al.*, 2010) as a search model. In order to solve the structure of α_1m by multiwavelength anomalous dispersion methods, a selenomethionyl derivative of α_1m, which contains four methionine residues, is being prepared.

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