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Recombinant bovine uteroglobin at 1.6 Å resolution: a preliminary X-ray crystallographic analysis

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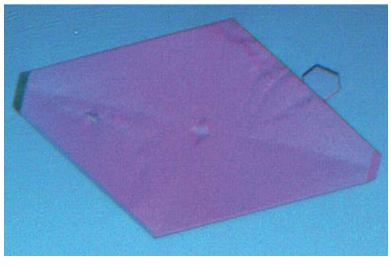


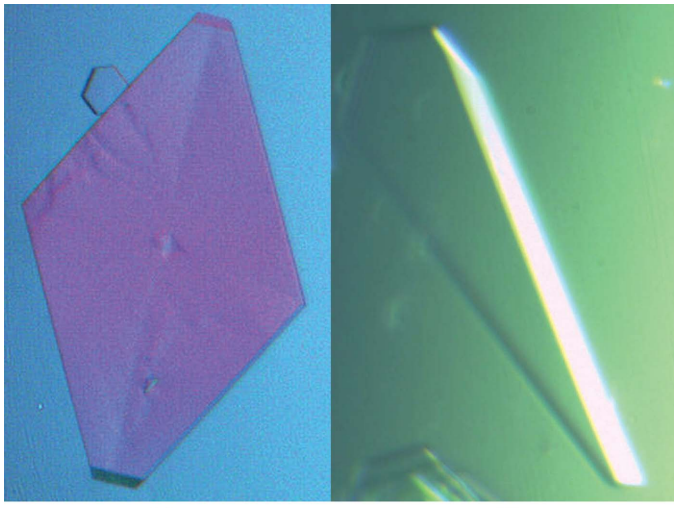
Uteroglobin (UG) is a conserved protein which is induced by progesterone and secreted by the epithelia of various mammalian reproductive and respiratory organs. Recombinant bovine uteroglobin (recbUG), consisting of 80 amino acids with a C-terminal His_6 tag, was overexpressed in *Escherichia coli* and purified. The protein was crystallized in two geometric forms, rhomboid and cuneate (wedge-shaped), by the hanging-drop vapour-diffusion method at 295 K. The rhomboid crystals diffracted to a maximum resolution of 1.6 Å using synchrotron radiation. These crystals belong to space group $P2_12_12$, with unit-cell parameters $a = 81.42$, $b = 82.82$, $c = 45.26$ Å, and contain four monomers per asymmetric unit. The cuneate crystals diffracted to 2.35 Å resolution using a rotating-anode generator. These crystals belong to space group $C222_1$, with unit-cell parameters $a = 43.39$, $b = 93.94$, $c = 77.30$ Å, and contain two molecules per asymmetric unit.

1. Introduction

Uteroglobin (UG) is a progesterone-dependent protein that is released into uterine secretions during the pre-implantation phase. It was first discovered using electrophoretic and immunological techniques as a major component of the blastocyst fluid and uterine seretion in the rabbit (Beier, 1966, 1968, 2000). Independently, Krishnan and Daniel identified the same protein in rabbit uterine fluid by ion-exchange chromatography and named it blastokinin (Krishnan & Daniel, 1967). Following its identification in the rabbit uterus, UG was also found in other organs (Beier *et al.*, 1975, 1978; El Etreby *et al.*, 1983). It is identical to the Clara cell 10 kDa protein (CC10), which is expressed in the lung (Singh *et al.*, 1988), and to protein-1 (P1) found in urine (Bernard *et al.*, 1992). UG has also been referred to as Clara cell phospholipid-binding protein, Clara cell secretory protein (CCSP), Clara cell 16 kDa protein (CC16) and polychlorinated biphenyl (PCB) binding protein (Mukherjee *et al.*, 1999). A nomenclature meeting, held during the symposium on the uteroglobin/Clara cell protein family, recommended the unifying generic name secretoglobin (SCGB) for all members of this gene family. The master gene is located on human chromosome 11, which contains related clusters of secretoglobin genes mapping to cytogenetic band 11q-12, or 280–320 cR on the radiation hybrid map (Klug *et al.*, 2000; Reynolds *et al.*, 2002). 'Secretoglobin' refers to all UG-like molecules with common characteristics such as secretion proteins, small globular size, α-helical and dimeric structure, but it avoids any organ-specific nomenclature.

During the past two decades, the isolation and characterization of many cDNAs encoding UGs has been reported, including those from the mouse (Ray *et al.*, 1993), pig (Gutierrez Sagal & Nieto, 1998), Syrian hamster (Dominguez, 1995), horse (Muller-Schottle *et al.*, 2002), cow (van der Decken *et al.*, 2005) and from humans (Singh *et al.*, 1988; Zhang *et al.*, 1997). These sequences reveal that native UG is a conserved protein, consisting of two identical 70-amino-acid subunits connected in an antiparallel orientation by two disulfide bridges, thus forming a hydrophobic cavity (Morize *et al.*, 1987; Umland *et al.*, 1994). The conservation of this structure implies an important physiological function (Miele *et al.*, 1994, 1987). Although many different functions have been proposed, the precise physio-

logical role of this protein has not been determined. It has been suggested that UG may have potent anti-inflammatory and immunomodulatory properties, *e.g.* through the inhibition of phospholipase A_2 activity (Levin *et al.*, 1986; Miele *et al.*, 1987). The protein has also been shown to inhibit human and rabbit phagocyte chemotaxis and phagocytosis (Vasanthakumar *et al.*, 1988). Mukherjee *et al.* (1982) suggested that UG could play a specific role in masking the antigenicity of developing embryos during implantation. From these results, it was suggested that UG may protect the developing embryo from maternal immunological attack, not only by binding to the antigenic determinants of embryonic cells, but also by impairing the migration of phagocytes and thus inhibiting phagocytosis (Mukherjee *et al.*, 1980).



(*a*) (*b*)

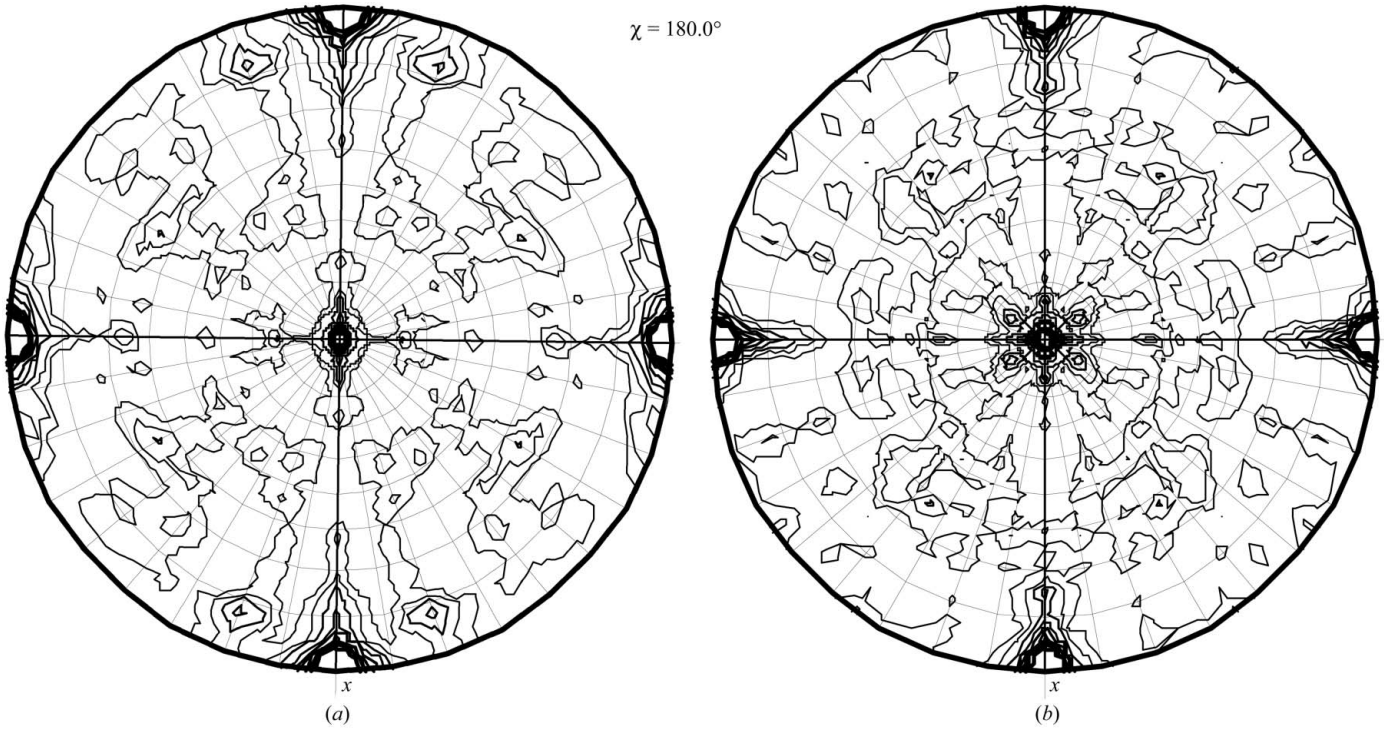
Figure 1
(*a*) Rhomboid recbUG crystal. (*b*) Cuneate recbUG crystal.

In order to gain further insight into the physiological role of UG, we would like to compare the sequence and structure of bovine UG with those of other mammalian species in order to study their level of conservation. Here, we report the bacterial expression, crystallization and preliminary X-ray crystallographic analysis of recombinant bovine uteroglobin.

2. Materials and methods

A cDNA fragment corresponding to the open reading frame of bovine uteroglobin (GenBank accession No. AY994053) was amplified by polymerase chain reaction (PCR) from a cDNA clone. The oligonucleotides were designed to introduce *Bgl*I and *Xho*I restriction-endonuclease sites at the initiation and termination codons, respectively. The PCR product was digested with *Bgl*I and *Xho*I, purified by agarose gel electrophoresis and cloned into the *Escherichia coli* expression plasmid pET26B+ containing T7 promoter, pelB-leader and a C-terminal His_6 tag (Novagen, Germany).

The histidine-tagged recbUG was expressed in the periplasm of *E. coli* strain BL21 (DE3). Purification was accomplished at room temperature using Ni–NTA metal-affinity columns (Qiagen, Germany) according to the method of Stocker *et al.* (2003). This step was followed by buffer-exchange into 25 m*M* Tris–HCl pH 7 using a Sephadex G25 column (PD10, Amersham Biosciences, Germany) according to the manufacturer's instructions. Further purification was carried out by anion-exchange chromatography using a Source 15Q PE 4.6/100 column (Amersham Biosciences, Germany). The recbUG was eluted at 130 m*M* NaCl using a linear gradient with 25 m*M* Tris–HCl, 1 *M* NaCl pH 7 as the second buffer. The final purification step was size-exclusion chromatography using PBS buffer, which was carried out using a Sephacryl S-100 HR (HiPrep 16/60) column (Amersham Biosciences, Germany). The purified protein was dialyzed overnight against 10 m*M* Tris pH 8.3, 0.5 m*M* EDTA and



(*a*) (*b*)

Figure 2
Self-rotation at κ section 180°. (*a*) Rhomboid recbUG crystal ($P2_12_12$). (*b*) Cuneate recbUG crystal ($C222_1$).

concentrated by ultrafiltration using a Nanosep 10k Omega microconcentrator (Pall Life Sciences, Germany). The protein concentration was determined at 280 nm by applying a theoretical molar extinction coefficient of 1400 M^{-1} cm^{-1}. The final concentration of the solution was 19.1 mg ml^{-1}. To determine whether the recombinant protein assembled into its native dimeric form, small amounts of the solution were fractionated by SDS–PAGE using a 5% stacking gel and an 18% resolving gel under different conditions. The recbUG was diluted either in a non-reducing loading buffer (without β-mercaptoethanol), or in a weakly reducing (2 mM DTT) or more strongly reducing [5%(v/v) β-mercaptoethanol] buffer. One group of samples was heated at 373 K for 5 min, whereas the other samples were maintained at 273 K. A 10 µg aliquot of diluted protein was loaded onto each lane of the gel and electrophoresis was performed at 180 V for 65 min. After electrophoresis the gel was stained with Coomassie Brilliant Blue G-250 (BioRad, Germany).

3. Results and discussion

Purified protein was crystallized using the hanging-drop vapor-diffusion method at 295 K. Thin crystalline plates of recbUG were obtained using a mixture of 2-propanol, sodium acetate and $CaCl_2$ as the reservoir solution. The hanging drops consisted of 1.5 µl protein solution and 1.5 µl reservoir solution. By adding PEG 4000 or 8000 to concentrations between 3 and 5%, two different crystal forms, rhomboid and cuneate (wedge-shaped), appeared reproducibly after about three weeks (Fig. 1). The reservoir solution (1 ml) for the rhomboid crystals was 100 mM sodium acetate pH 5.1, 200 mM $CaCl_2$, 20% 2-propanol and 5% PEG 8000. For the cuneate crystals, the reservoir solution (1 ml) was 100 mM sodium acetate pH 4.9, 200 mM $CaCl_2$, 17% 2-propanol and 3% PEG 4000.

Both crystal forms were mounted in small loops using glycerol as a cryoprotectant [final concentration 30%(v/v)]. After mounting the crystals, they were immediately flash-cooled to 100 K in a nitrogen stream (Oxford Cryosystems, UK). X-ray data were collected in-house and at beamline X13 of the EMBL/DESY facility in Hamburg. For the rhomboid crystals, we collected a complete data set with a maximum resolution of 1.6 Å using synchrotron radiation (λ = 0.803 Å and a MAR CCD imaging plate). For the cuneate crystals, a data set was recorded using radiation from an in-house rotating copper-anode generator (Bruker AXS FR591 with a MAR 345dtb image-plate system).

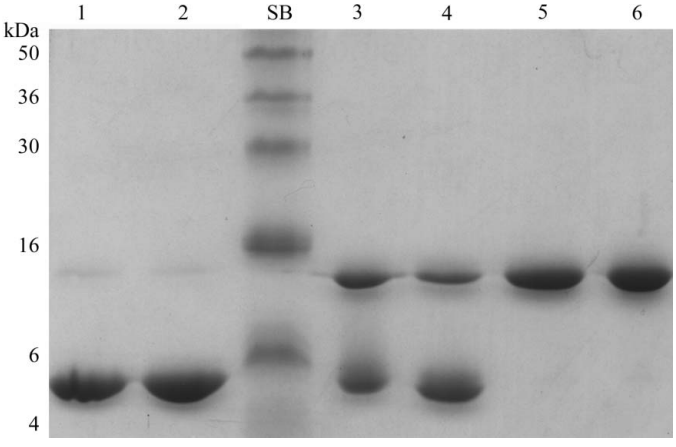


Figure 3
Coomassie-stained SDS–PAGE gel (18%) showing purified recbUG under different reducing and non-reducing conditions, in order to determine stability and purity. Lane 1, recbUG + 5%(v/v) β-ME (373 K, boiled for 5 min); lane 2, recbUG + 5%(v/v) β-ME (297 K); lane 3, recbUG + 2 mM DTT (373 K, boiled for 5 min); lane 4, recbUG + 2 mM DTT (297 K); lane 5, recbUG (373 K, boiled for 5 min); lane 6, recbUG (297 K). SDS–PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; β-ME, β-mercaptoethanol; DTT, dithiothreitol.

Table 1
Data-collection statistics.

	Rhomboid	Cuneate
Space group	$P2_12_12$	$C222_1$
Unit-cell parameters (Å, °)	a = 81.42, b = 82.82, c = 45.26, $\alpha = \beta = \gamma$ = 90	a = 43.39, b = 93.94, c = 77.30, $\alpha = \beta = \gamma$ = 90
Matthews coefficient (Å^3 Da^{-1})	2.2	2.2
Solvent content (%)	42.4	44.1
Unit-cell volume (Å^3)	305197	315079
No. of molecules in AU	4	2
Observed reflections	220794	48675
Unique reflections	40012	6808
Wavelength (Å)	0.803	1.5414
Resolution range (Å)	82–1.6	20–2.35
Completeness (%)	97.0	98.8
R_{merge} (%)	9.2	8.1

The data were processed and scaled with *XDS* (Kabsch, 1993) and the self-rotation functions (Fig. 2) were calculated with *CCP*4 (Collaborative Computational Project, Number 4, 1994). The rhomboid crystal form was shown to belong to space group $P2_12_12$, with unit-cell parameters a = 81.42, b = 82.82, c = 45.26 Å. Assuming the presence of two molecules in the asymmetric unit, a Matthews coefficient of 4.3 Å^3 Da^{-1} was calculated. Since recbUG forms a dimer in solution and four molecules resulted in a Matthews coefficient of 2.2 Å^3 Da^{-1}, we concluded that there were four molecules per asymmetric unit. Additionally, the self-rotation showed a twofold axis, which could show the symmetry between two dimers. Analysis of the cuneate crystal form indicated an orthorhombic centred space group $C222_1$, with unit-cell parameters a = 43.39, b = 93.94, c = 77.30 Å. Assuming the presence of two molecules in the asymmetric unit, a Matthews coefficient of 2.2 Å^3 Da^{-1} was calculated. Again, a twofold axis was shown in the self-rotation function. The data statistics are summarized in Table 1.

Fig. 3 shows a Coomassie-stained SDS–PAGE gel (18%), which demonstrates the stability of recbUG. The protein was stored for three weeks at 277 K and no degradation was visible. Additionally, a strong denaturating agent such as β-mercaptoethanol was required to reduce its dimeric structure completely. The protein is not affected by heat, as shown in lanes 1, 3 and 5 where the protein has been boiled before loading. There is no visible difference between these samples and the others which were not subjected to boiling.

Structural determinations are in progress. The structure of recbUG will be compared with existing UG structures in order to provide more information about the physiological functions of this conserved protein.

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